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Transport a metabolismus radioaktivně značených cytokininů v rostlinných buňkách a pletivech

Transport and Metabolism of Radio-Labelled Cytokinins in Plant Cells and Tissues

DIPLOMOVÁ PRÁCE

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Prohlášení

Prohlašuji. že jsem závěrečnou práci zpracoval samostatně a že jsem uvedl všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze dne 12. 6. 2020 .....
Daniel Nedvěd, v. r.

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Abstract

Cytokinins are a large group of phytohormones. Since their discovery in the 1950s, they have shown to play a pivotal role in plant physiology. Most studies so far focused on cytokinin action mechanisms and their metabolic regulation. Identification of AtABCG14 and AtPUP14 as cytokinin-specific membrane carriers brought researchers' attention to cytokinin membrane transport, too.

In this thesis, we performed experiments with radio-labelled cytokinin tracers. We show that *trans*-zeatin and isopentenyladenine, two major biologically active cytokinins, are readily transported across the plasma membrane in tobacco BY-2 cell suspension. Making use of mathematical modelling, we show that BY-2 cells possess a membrane transport system with an affinity toward cytokinins. Next, we show that *atabcg14* and *atpup14* mutations affect cytokinin metabolism in *Arabidopsis thaliana* plants.

Keywords: cytokinin, *Arabidopsis thaliana*, tobacco BY-2 cell lines, membrane transport, purine permease, ATP-binding cassette, radio-labelling

Abstrakt

Cytokininy jsou významnou skupinou fytohormonů. Od svého objevu v 50. letech 20. století se ukázaly být jedním z klíčových regulátorů rostlinné fyziologie. Jejich výzkum byl dosud zaměřen na mechanismus jejich účinku a na metabolickou regulaci jejich působení. Poté, co byly proteiny AtABCG14 a AtPUP14 identifikovány jako jejich membránové přenašeče, se začal v souvislosti s cytokininy zkoumat také jejich membránový transport.

V této práci jsme v řadě experimentů využili sledování radioaktivně značených cytokininů. Ukazujeme, že *trans*-zeatin a isopentenyladenin, dva hlavní biologicky účinné cytokininy, jsou v tabákových buněčných liniích BY-2 přenášeny přes plasmatickou membránu. S využitím matematického modelování ukazujeme, že se na membránách buněk BY-2 nachází transportní systém vykazující afinitu vůči cytokininům. Dále ukazujeme, že mutace *atabcg14* a *atpup14* mají vliv na metabolismus cytokininů v rostlinách *Arabidopsis thaliana*. [Anglicky]

Klíčová slova: cytokinin, *Arabidopsis thaliana*, tabákové buněčné linie BY-2, membránový přenos, purinová permeasa, přenašeč ABC (z anglického “ATP-binding cassette”), radioaktivní značení

Table of Contents

1 Abbreviation and Symbol List

1.1 Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
ABC	ATP-binding cassette
Ade	Adenine
Ado	Adenosine
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
At	*Arabidopsis thaliana*, mouse-ear cress
ATP	Adenosine triphosphate
AUC	Area under a curve
BAP	6-Benzylaminopurine
BY-2	tobacco cultivar “Bright yellow 2”
CI	Chemical ionization
CKX	Cytokinin oxidase/dehydrogenase
CoA	Coenzyme A
Col-0	Columbia, an *Arabidopsis thaliana* ecotype
CPPU	*N*-(2-Chloro-4-pyridyl)-*N’*-phenylurea
CYP	Cytochrome P450
cZ	*cis*-Zeatin
cZ9G	*cis*-Zeatin N^9-glucoside
DEAE	Diethyalaminoethyl
DHZ	Dihydrozeatin
DMAPP	Dimethylallyl pyrophosphate
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DPU	Diphenylurea
EI	Electron ionization
ELISA	Enzyme-linked immunoassay
ENT	Equilibrative nucleoside transporter
ESI	Electrospray ionization
FAD	Flavin adenine dinucleotide
GC	Gas chromatography
HMBPP	(*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate

HMG	3-Hydroxyl-3-methylglutaryl
HPLC	High-performance liquid chromatography
IAA	Indole-3-acetic acid
IAC	Imunoaffinity chromatography
IEX	Ion exchange
iP	Isopentenyladenine
iP9G	Isopentenyladenine N^9-glucoside
iPR	Isopentenyladenine riboside
iPRDP	Isopentenyladenine riboside diphosphate
iPRMP	Isopentenyladenine riboside monophosphate
iPRTP	Isopentenyladenine riboside triphosphate
IPT	Isopentenyl transferase
LC	Liquid Chromatography
LOG	“Lonely guy”, cytokinin phosphoribohydrolase
MES	2-(*N*-morpholino)ethanesulfonic acid
MS	Mass spectroscopy
MS/2	“Half Murashige-Skoog”, a medium used to grow *Arabidopsis* plants
mT	*meta*-Topolin
NASC	Eurasian *Arabidopsis* stock centre, an *Arabidopsis* seed database
NBD	Nucleotide-binding domain
Nt	*Nicotiana tabacum*, tobacco
OE	Overexpressor
Os	*Oryza sativa*, Asian rice
oT	*ortho*-Topolin
PIN	Pin-formed, a membrane transporter family involved in polar auxin transport
pT	*para*-Topolin
PUP	Purine permease
RIA	Radioimmunoassay
RISR	Relative internal standard response
RPM	Revolutions per minute, a unit expressing shaking or centrifugation frequency
SIM	Single-ion monitoring
SPE	Solid-phase extraction
TDZ	Thidiazuron
TMD	Transmembrane domain

tRNA	Transfer ribonucleic acid
tZ	*trans*-Zeatin
tZ7G	*trans*-Zeatin N^7-glucoside
tZ9G	*trans*-Zeatin N^9-glucoside
tZR	*trans*-Zeatin riboside
tZRDP	*trans*-Zeatin riboside diphosphate
tZRMP	*trans*-Zeatin riboside monophosphate
tZRTP	*trans*-Zeatin riboside triphosphate
UGT	Uridinediphosphate-glucuronosyltransferase, a glycosylation enzyme
VPE	Vapour-phase extraction
WT	Wild type
Zm	*Zea mays*, maize

1.2 Symbols

c or []	Concentration	$mol \times L^{-1}$
J	Diffusion flux	$mol \times m^{-2} \times s^{-1}$
K_A	Acidity constant	*dimensionless*
K_I	Inhibition constant	$mol \times L^{-1}$
K_M	Transporter affinity constant	$mol \times L^{-1}$
NSR	Non-saturable component of the transport rate	$mol \times s^{-1}$
t	Time	s
v	Transport rate	$mol \times L^{-1} \times s^{-1}$
V_{max}	Maximal (limit) transport rate	$mol \times L^{-1} \times s^{-1}$
ρ	Cell suspension density	$(10^6 \text{ cells}) \times L^{-1}$

2 Introduction

2.1 Phytohormones

2.1.1 The Concept of Hormonal Regulation in Plants

To cope with limitations provided by the sessile lifestyle, such as lack of behavioural reactions, and to thrive under varied environmental conditions, the plants possess complex mechanisms to regulate their growth, development and metabolism. A lot of such processes are mediated by chemical compounds called phytohormones or plant hormones. As reviewed in [1], the exact definition of phytohormones has not been introduced. While it has been stated that phytohormones are substances capable of promoting or inhibiting plant physiological processes, effective at relatively low

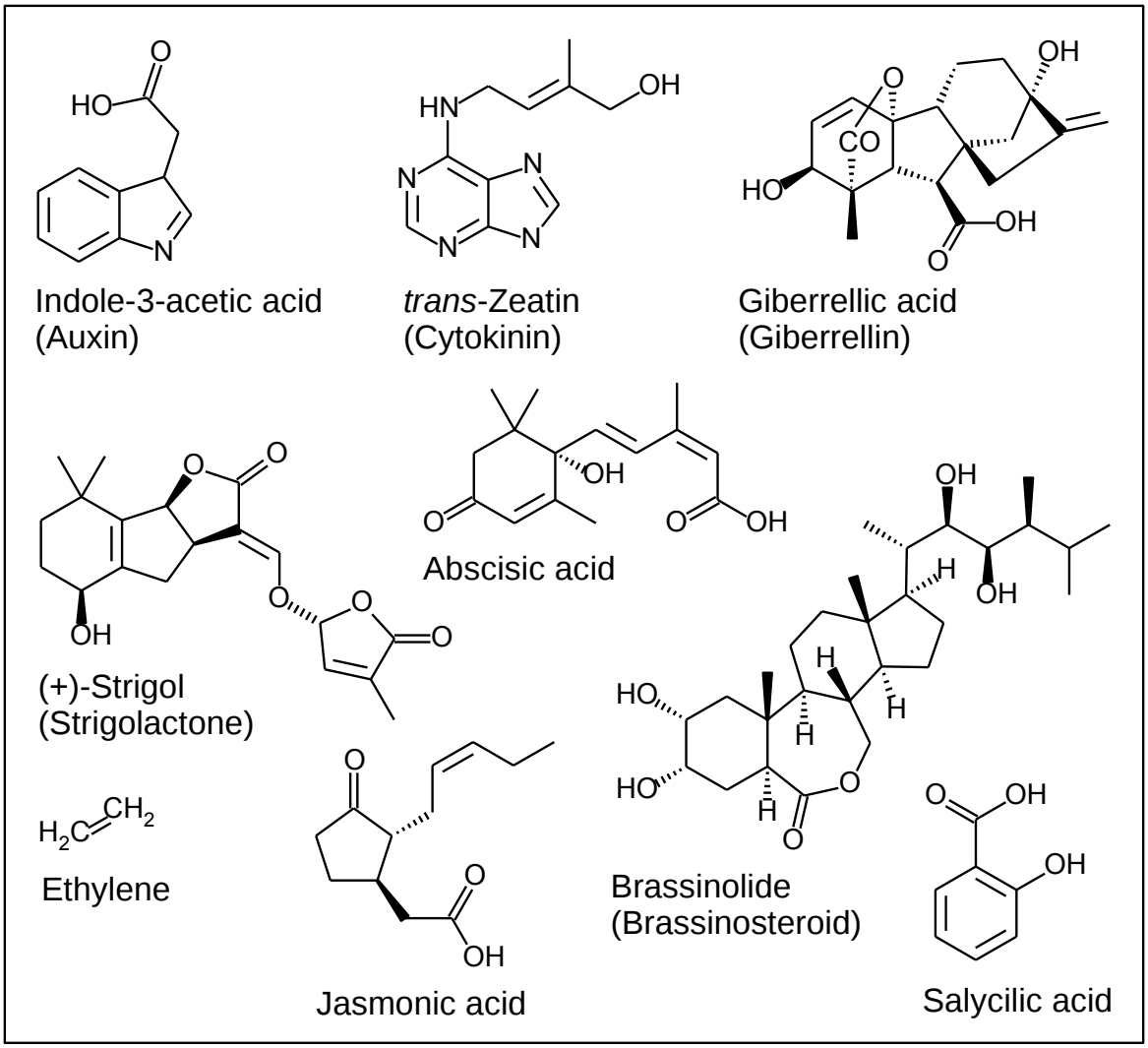

Figure 1: Structural diversity of phytohormones. Those hormones that belong to a larger group of structurally or functionally related compounds are denoted with the name of this group in parentheses.

concentrations (typically within the range of units or tens of μmol×L^{-1}), some other questions, such as if the term should apply to synthetic compounds as well, remain unsolved [1], [2].

The existence of such molecules in plants was proposed decades before their actual discovery and before the concept of hormonal regulation arose. Notably, Charles and Francis Darwin advocated for the existence of compounds that would be transported between the root and the shoot and that would induce plant movements in direct response to light and gravity, phenomena known as phototropism and gravitropism, respectively [3]. Later research showed that these processes are indeed mediated by regulatory molecules, namely by plant hormones known as auxins [4], [5].

Nowadays, the known phytohormones are divided into several classes, including aforementioned auxins [6], [7], cytokinins, that will be the main topic of this thesis, gibberellins [8], [9], brassinosteroids [10], [11] and strigolactones [12], [13]. Phytohormones belonging to one of these classes may have in common both function in plants and structural features. Apart from them, there are also phytohormones which do not belong to any group. They include ethylene [14], abscisic acid [15], [16], jasmonic acid [17], [18] and salicylic acid [19], [20]. Structures of various phytohormones can be seen in Figure 1. Recently, the signalling function of some plant peptides has been observed as well [21].

2.1.2 Comparison of Plant and Mammalian Hormones

Initially, the phytohormones were supposed to obey a general model of action, wherein they are transported from the site of synthesis to the site of action via vascular tissues. This would be an analogy of the mammalian endocrine system, thus the term “hormone” was adopted [22]. However, the following decades of research have shown that the plant and mammalian hormones have little in common.

As seen in Figure 1, plant hormones are typically low-molecular, whereas, in mammalian endocrine regulation, large peptides and proteins are involved as hormones as well. Even so, one may find that both plant and mammalian hormones include steroid compounds, substances derived from fatty acids (compare biosynthesis of jasmonic acid in plants [18] and arachidonic acid derivatives in mammals [23]) or amino acid metabolites; for instance, tryptophan is a common precursor of both indole-3-acetic acid (IAA), the most abundant auxin [24], and mammalian melatonin, secreted by pineal gland [25].

In contrast to mammals, plants do not form tissues specialized to hormone production and conversely, all phytohormones can be synthesized in almost any cell, though the rates of their production may differ.

Contrary to the original belief mentioned above, the classical “synthesis-transport-action” isn’t sufficient for all hormone-mediated processes in plants. Along with being distributed via long-distance transport, plant hormones can also affect cells in the paracrine manner, where the target cells are adjacent to the site of synthesis, in the autocrine manner, where the hormone affects the same cell in which it has been produced [22], [26].

While most mammalian hormones can be associated with their particular effects on the organism, things are more complicated when it comes to plants. Phytohormones rarely act on their own; instead, they are often subject to crosstalk, be it with another hormone, a different kind of signal molecule or environmental stimuli. This can be shown on the complicated relationship between auxins and cytokinins, as these two major groups of phytohormones regulate each other’s synthesis, metabolism, transport and signalling pathway [27]. Besides, a hormone-mediated process can also depend on the type and age of the tissue wherein it takes place. Therefore, we can say that a certain plant hormone typically affects diverse processes and that a certain process is a result of multiple signal pathways interaction [26], [28], [29].

All thing considered, plant and mammalian hormones are unlike substances. All they have in common is that they act as chemical messengers in their respective organisms.

2.1.3 Plant Hormones Outside of Plant Biology Studies

The interest in phytohormones reaches beyond the area of basic research in plant physiology. To begin with, salicylic acid has been used as an anti-inflammatory agent for centuries, be it in a form of willow bark and leaves or as a drug known as aspirin [19], [30]. As reviewed in [31], the effects of jasmonic acid and its derivatives, mainly its methyl ester, on the human organism and their potential use in medicine have been profoundly studied. Cytokinins were studied in relation to the antioxidant protection of mammalian cells [32], which led to their application in cosmetics [33].

It is not surprising that the knowledge of plant hormones has many applications in agriculture and horticulture. For instance, auxins function as a base of many herbicides [34]. Ethylene promotes ripening in some fruits, even in those that have been already picked. In large-scale agriculture, it is common to pick certain fruits, notably bananas, unripe and transport them in this state. Eventually, they are artificially ripened with the

exogenous application of ethylene. Such a procedure is usually more economical than harvesting ripe fruits, as the damage caused by transport is minimized and the ripening process may be regulated according to customers' demand [35]. The hormonal treatment, most notably the exogenous application of gibberellins, is also used for seedless fruit production [36], [37].

2.2 Structure and Chemical Properties of Cytokinins

2.2.1 Structural Diversity of Naturally-Occurring and Synthetic Cytokinins

As stated above, cytokinins, both naturally-occurring and synthetic, are a major group of plant hormones. Cytokinin signalization is involved in several physiological processes and plant interactions with the environment [38]. Similarly to most plant hormones described so far, cytokinins are low-molecular. All naturally-occurring cytokinins may be described as derivatives of adenine. The said derivatization occurs at N^6 atom, where the hydrogen residue, present in adenine, is substituted by a side chain. Based on their chemical structure, the N^6-side chains can be classified as either isoprenoid, containing a five-carbon branched moiety, or aromatic, containing a benzene ring. Both kinds of side chain may be further derivatized [39], [40]. The same classification applies for cytokinins as whole molecules, too.

The first naturally-occurring cytokinin was discovered by Letham et. al [41]. Because it was isolated from maize, or *Zea mays* in Latin, the authors dubbed it zeatin. Its side chain is of an isoprenoid character, consisting of an isoprenoid moiety with a terminal hydroxyl group [42]. Considering stereochemistry of such a compound, it follows that zeatin can adopt either *trans* or *cis* configuration on the side-chain double bond. Both *trans*-zeatin (tZ) and *cis*-zeatin (cZ) occur in plants as and they are treated as two individual substances [43]. While tZ is a biologically active cytokinin in all plants, the activity of cZ differs in various plant species. In *Arabidopsis thaliana* (or mouse-ear cress), the main model plant organism, cytokinin receptors display much greater affinity toward tZ than toward cZ [44]. Other isoprenoid cytokinins include isopentenyladeinine (iP), whose side chain consists of an isopentenyl moiety without further derivatization, and dihydrozeatin (DHZ), a product of formal reduction of the double bond present in the side chain of either tZ or cZ [39], [40].

Naturally-occurring aromatic cytokinins comprise 6-benzylaminopurine (BAP), whose side chain consists of a benzyl residue, and its derivatives. Formal

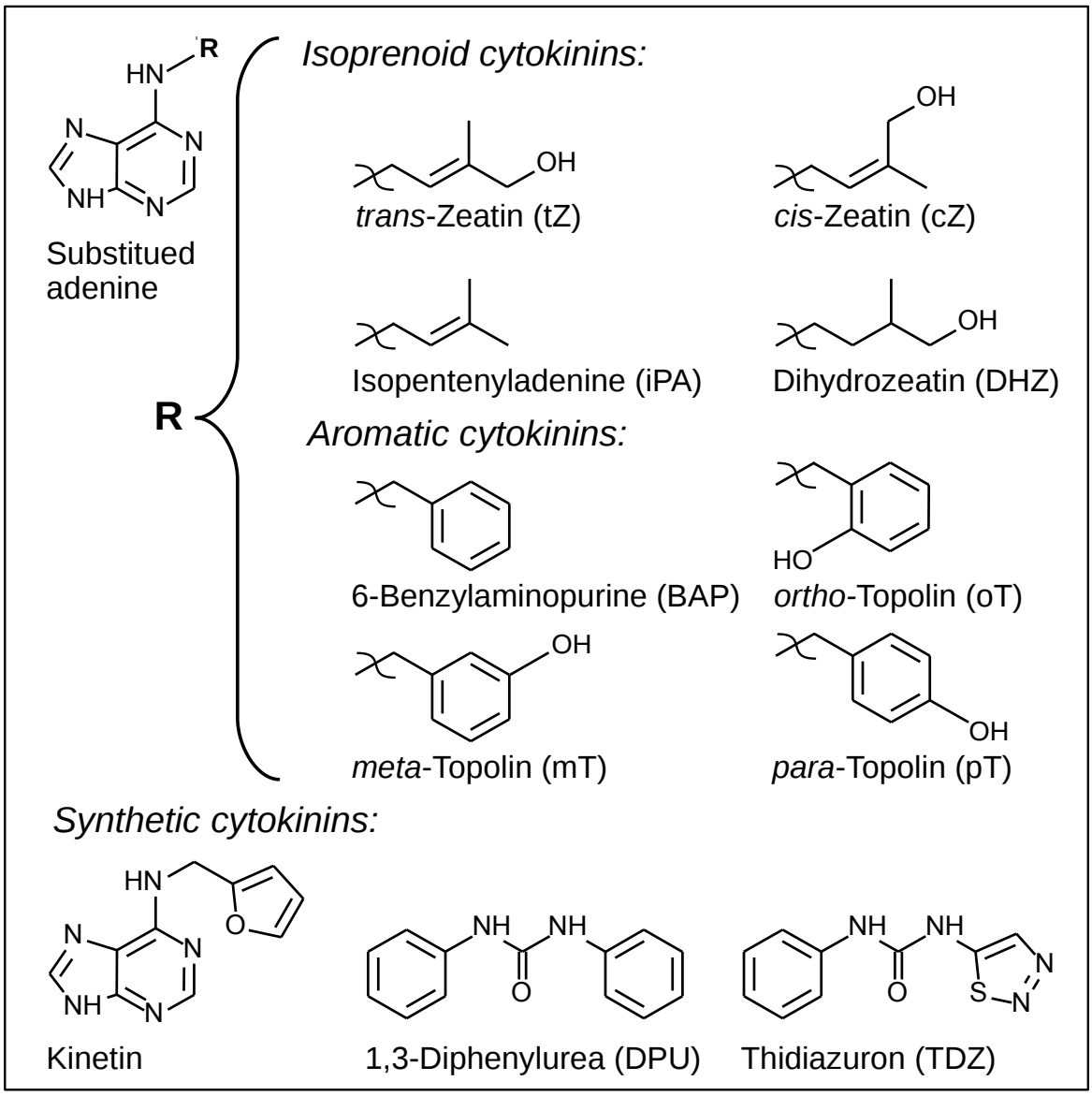

Figure 2: Structural diversity of cytokinins. Naturally-occurring cytokinins are derivatives of adenine (the substitution site is marked in the upper left corner). Based on the substituent character, such cytokinins are classified either as isoprenoid or aromatic. Synthetic cytokinins can be derivatives of either adenine, such as kinetin, or 1,3-diphenylurea (which is considered a cytokinin as well). Common cytokinin abbreviations are given in parentheses.

monohydroxylation of the aromatic ring within such a side chain yields three distinct products, collectively called topolins. Such a name was derived from “topol”, the Czech word for the poplar tree (*Populus x canadensis*), from which the topolins were originally purified. According to the hydroxyl position, the three BAP derivatives are classified as either *ortho*-, *meta*- or *para*-topolin [39], [45]–[47].

Synthetic cytokinins may be derivatives of adenine as well. A striking example of such a compound is kinetin, the first substance with cytokinin activity described. Its discovery dates back to the '50s when C. Miller and F. Skoog proposed the existence of a cell division-promoting factor in coconut milk. Later, they managed to isolate such a

compound by autoclaving animal DNA. They proposed the name kinetin, reflecting its effect on cell division. Eventually, kinetin was revealed to be N^6-furfuryladenine, with an aromatic side chain [48]–[50]. Some other synthetic cytokinins are formal derivatives of phenylurea, e.g. diphenylurea (DPU), thidiazuron (TDZ) and *N*-(2-chloro-4-pyridyl)-*N'*-phenylurea (CPPU) [39]. While TDZ and its hydroxylated derivatives are recognized by cytokinin receptors and cytokinin-specific enzymes [51]–[53], DPU rather prevents the degradation of endogenous cytokinins [54]. Structural formulas of various cytokinins are given in Figure 2.

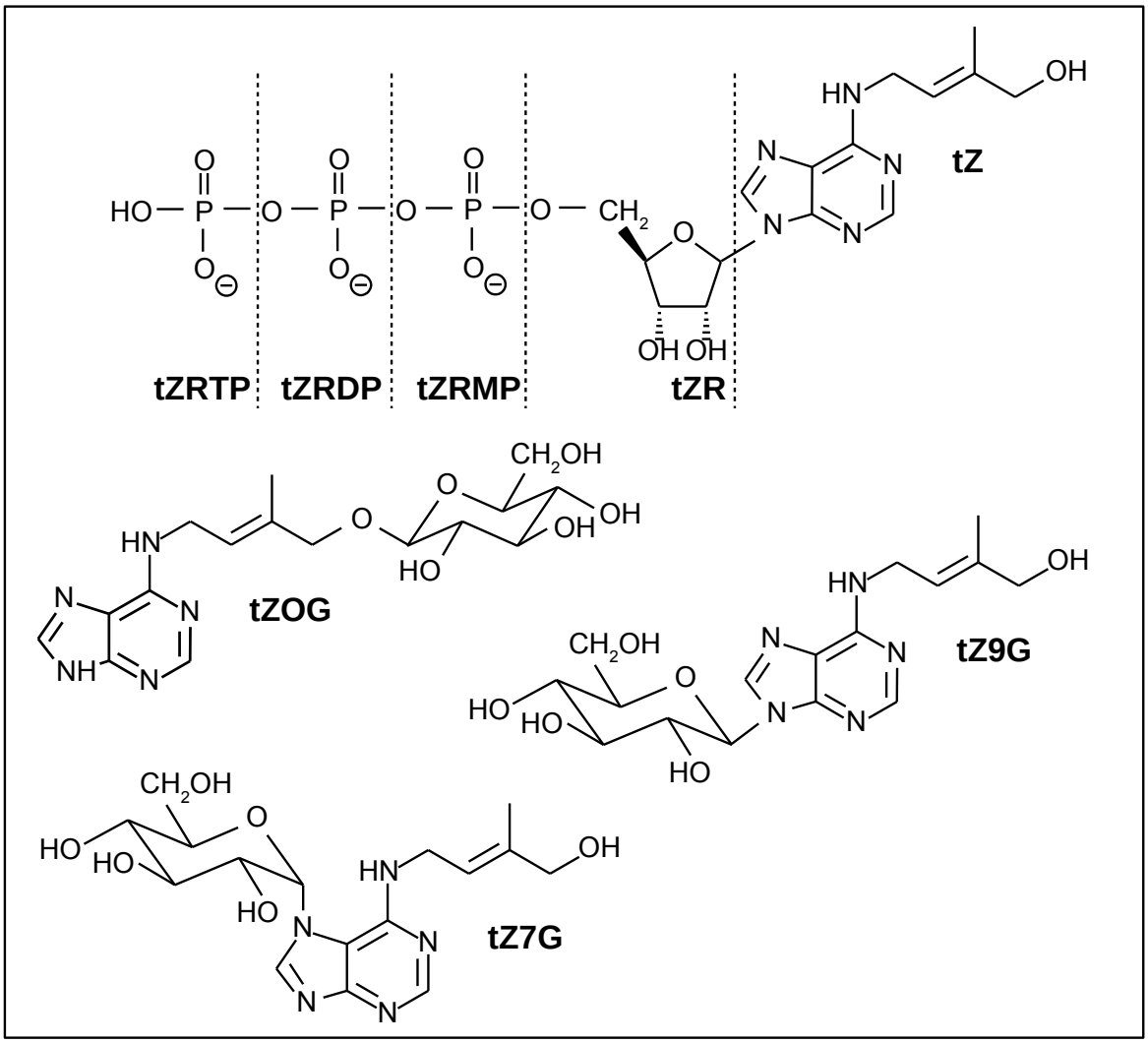

Figure 3: Various cytokinin forms are shown on an example of trans-zeatin (tZ). Conjugation of the tZ free base with a ribose moiety via an N^9-glycosidic bond yields trans-zeatin riboside (tZR). Further tZR fosforylation yields trans-zeatin monophosphate (tZRMP), diphosphate (tZRDP), and triphosphate (tZRTP). Glycosylation of tZ at the side-chain O atom, via an O-glycosylic bond, yields trans-zeatin O-glucoside (tZOG). Glycosylation of tZ at N^7 and N^9 atom yields trans-zeatin N^7-glucoside (tZ7G) and trans-zeatin N^9-glucoside, respectively. These forms exist for other cytokinins as well.

2.2.2 Variety of Cytokinin Forms and Roles

The adenine-like cytokinins discussed above are classified as free bases. It means that their adenine core is not derivatized anywhere but on the N^6 atom. The free bases are generally considered the biologically active forms of cytokinins [40]. Other forms of cytokinins include cytokinin nucleosides (or ribosides), nucleotides (or riboside monophosphates) and glucosides.

Nucleosides generally consist of a base attached to a pentose moiety via an *N*-glycosylic bond. In the case of cytokinins, the adenine derivatives are conjugated with ribose. The ribosylation occurs on the N^9 atom. In cytokinin nucleotides, the ribose moiety is further linked to a phosphate group via a phosphodiester bond. Cytokinin glucosides are conjugates of free bases with a glucose moiety either via an *N*-glycosylic bond, which may occur in multiple positions, or an *O*-glycosylic bond, which is confined to the hydroxylic group of tZ, cZ, and DHZ side chains. Cytokinin nucleosides and glucosides are often referred to as transport and storage forms of cytokinins, respectively. Cytokinin nucleotides appear as intermediates in cytokinin biosynthesis (see 2.3.1) [39], [40]. Different cytokinin forms, demonstrated on tZ, are depicted in Figure 3.

Cytokinin nucleotides can be also found incorporated into tRNAs of most organisms, except for Archaea. Their presence stems from enzymatic modifications of adenine bases that already make part of tRNA molecules. Their function is to stabilize codon-anticodon interactions during translation [55], [56].

2.3 Cytokinin Metabolism

2.3.1 Biosynthesis of Biologically Active Cytokinins

The biosynthesis of isoprenoid cytokinins tZ and iP begins by conjugation of adenosine monophosphate (AMP), diphosphate (ADP) or triphosphate (ATP) with an activated isoprenoid moiety – either dimethylallyl pyrophosphate (DMAPP) or hydroxymethylbutenyl pyrophosphate (HMBPP). Such reactions yield iP and tZ nucleosides, respectively, bearing the corresponding number of phosphate groups [40], [57].

In plants, DMAPP is produced via two distinct pathways. The mevalonate pathway comprises of subsequent condensation of three acetyl-CoA molecules, yielding acetoacetyl-CoA and 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). The latter is reduced to mevalonate. Two phosphorylation steps follow, yielding mevalonate 5-phosphate and

mevalonate 5-diphosphate. Mevalonate 5-diphosphate is converted to isopentenyl pyrophosphate (IPP) through decarboxylation and dehydration. Eventually, DMAPP is formed as a product of a reversible isomerization reaction from IPP. This pathway is common to all eukaryotes and is localized in the cytoplasm [58], [59]. On the other hand, the MEP pathway, named after 2-*C*-methyl-D-erythritol 4-phosphate, is found in bacteria and plastids only. DMAPP is produced by a series of reactions from pyruvate and D-glyceraldehyde 3-phosphate. Since mevalonate is not formed, the MEP pathway is also called mevalonate-independent. One of the MEP pathway intermediates is HMBPP, an alternative source of the cytokinin isoprenoid side chain [59]–[61].

The conjugation between an adenine nucleotide and an activated isoprenoid moiety is catalysed by isopentenyl transferases (IPTs). Such a reaction was first found in *Dictyostelium discoideum*, a unicellular eukaryote [62]. Subsequently, the *IPT* gene was identified in *Agrobacterium tumefaciens*, a micro-organism known mainly as a plant pathogen [63]. With the use of bioinformatics tools, nine *IPT* homologues were identified in *Arabidopsis*, denoted *AtIPT1-9* [64]. It was shown that AtIPTs prefer ADP or ATP (but not AMP) and DMAPP, rather than HMBPP, as substrates [65]. Further studies revealed that AtIPTs differ in their subcellular localization, tissue specificity and roles in cytokinin metabolism. AtIPT1, 3, 5 and 8 are localized to plastids and they utilize DMAPP produced via the MEP pathway. AtIPT4 and 7 are localized to cytoplasm and mitochondria, respectively, and they utilize DMAPP produced via the mevalonate pathway [66]. AtIPT2 and 9 catalyse the transfer of the prenyl group to tRNA, rather than to free adenine nucleoside phosphates. Being localized to the cytoplasm, they make use of DMAPP produced via the mevalonate pathway. It was proposed that tRNA prenylation by AtIPT2 and 9, followed by hydroxylation and hydrolysis, is an important mechanism of free cZ biosynthesis [66]–[68]. This model is further supported by the relatively high occurrence of cZ-like nucleotides in plant tRNAs [69]. The role of AtIPT6 is yet to be examined [40], [57], [66].

Initial products of non-tRNA IPTs are isopentenyladenine riboside diphosphate (iPRDP) or triphosphate (iPRTP). These iP-type nucleoside phosphates can be converted to their corresponding tZ-type analogues via *trans*-hydroxylation. Such a reaction is catalysed by cytochrome P450 monooxygenases of the CYP735A subfamily. In *Arabidopsis*, two genes participate in iP-type nucleoside phosphates *trans*-hydroxylation, *CYP735A1* and *2*. Both of these prefer iPRDP and isopentenyladenine riboside monophosphate (iPRMP; i.e. a product of iPRTP and iPRDP defosforylation) as substrates, though they are capable of

converting iPRTP as well. Conversely, they do not *trans*-hydroxylate isopentenyl riboside (iPR) nor free iP [70]. The respective products of *CYP735A1* and *2* are called *trans*-zeatin riboside triphosphate (tZRTP), diphosphate (tZRDP) and monophosphate (tZRMP).

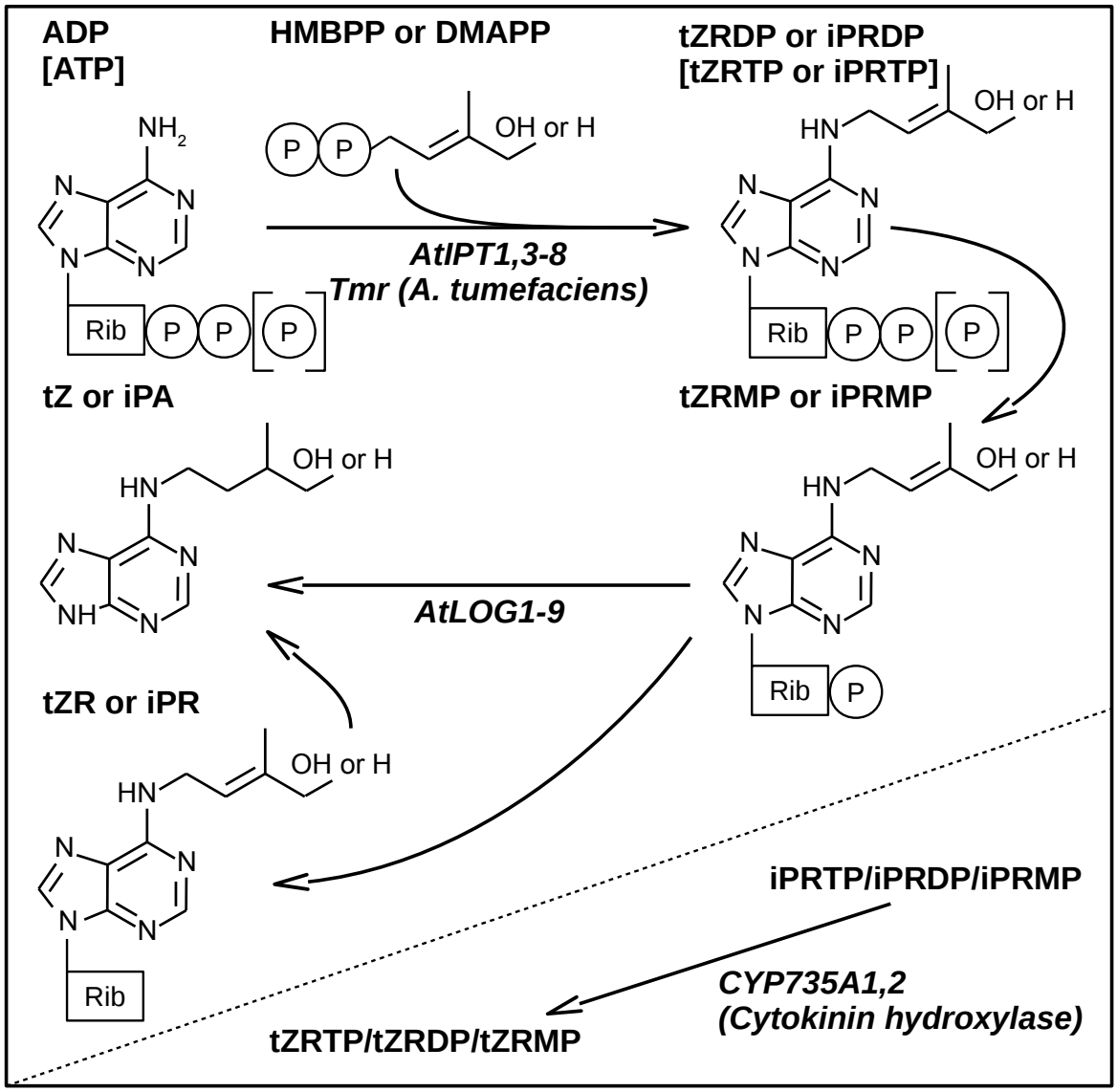

Figure 4: Basic biosynthesis scheme of trans-zeatin (tZ) and isopentenyladenine (iP). An adenine nucleotide, such as adenosine diphosphate (ADP) is prenylated. The side chain donor can be either (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) or dimethylallyl pyrophosphate (DMAPP). The reaction is catalysed by isopentenyl transferases (IPTs), such as AtIPT1,3-8 or Tmr. The initial product of this reaction is trans-zeatin riboside diphosphate (tZRDP) or isopentenyladenine riboside diphosphate (iPRDP), respectively. The isoprenoid side chain can be also transferred to adenosine triphosphate (ATP), as shown in brackets. In such a case, trans-zeatin riboside triphosphate (tZRTP) or isopentenyladenine riboside triphosphate (iPRTP) are formed. Cytokinin riboside phosphates are dephosphorylated to corresponding riboside monophosphates. These can be directly converted to the respective free bases. Such a reaction is catalysed by "Lonely guy" enzyme (cytokinin-activating phosphoribohydrolase), AtLOG1-9. Alternatively, cytokinin riboside phosphates can be dephosphorylated and deribosylated in two subsequent steps. As shown in the bottom right corner, iP ribosides can be converted to those of tZ via CYP735A1 and 2. This is a preferred way of tZ-type cytokinins in plants, whose IPTs have low affinity to HMBPP in general. Rib: ribose moiety, P: phosphate group. For complete structures, refer to Figure 3.

While *Arabidopsis* IPTs use DMAPP as the only source of the prenyl group for cytokinin biosynthesis, an IPT identified in *Agrobacterium*, denoted as Tmr, catalyses the transfer of both DMAPP and HMBPP to AMP. When infecting plants, *Agrobacterium* possesses an ability to transfer a portion of its genetic code, or the T-DNA localized within so-called Ti plasmid, into the host's genome. (On a side note, this ability has been readily used as a powerful tool in plant genetic engineering [71]). The *Tmr* gene is a part of the Ti plasmid and after the infection, it is expressed with the host plant cells. The reaction catalysed by Tmr directly produces tZRMP, which is readily converted to the biologically active tZ [72]. The *trans*-hydroxylation step, regulated by auxins [70], is skipped and the infected plant becomes impaired in phytohormone homoeostasis control. The uncontrolled biosynthesis of tZ-type cytokinins eventually leads to the formation of a tumour [72].

The last step of cytokinin biosynthesis consists in the conversion of cytokinin riboside monophosphates to biologically active free bases. Originally, a model consisting of two subsequent enzymatic reactions had been proposed. According to this model, the cytokinin nucleotides are substrates of 5'-nucleotidases, which catalyses the hydrolytic cleavage of the 5'-phosphoester bond, yielding the corresponding cytokinin nucleoside [73]. Next, the *N*-glycosylic bond of the cytokinin nucleoside is hydrolysed by a nucleosidase, yielding the free base [74]. Later, another pathway was found in rice shoot meristem, where cytokinin nucleotides are directly converted to the free bases via a single enzymatic reaction. During such a reaction, the *N*-glycosylic bond of a nucleotide is hydrolysed, yielding the corresponding free base and ribose 5'-monophosphate. Mutations in genes coding these enzymes often caused that only one stamen and no pistils were present in flowers. Hence, the gene family was dubbed "Lonely Guy" (*LOG*) [75]. Further analysis showed that the *Arabidopsis* genome harbours nine *LOG* genes (*AtLOG1 – 9*) that are at least partly redundant in their functions, as single-gene mutants do not exhibit

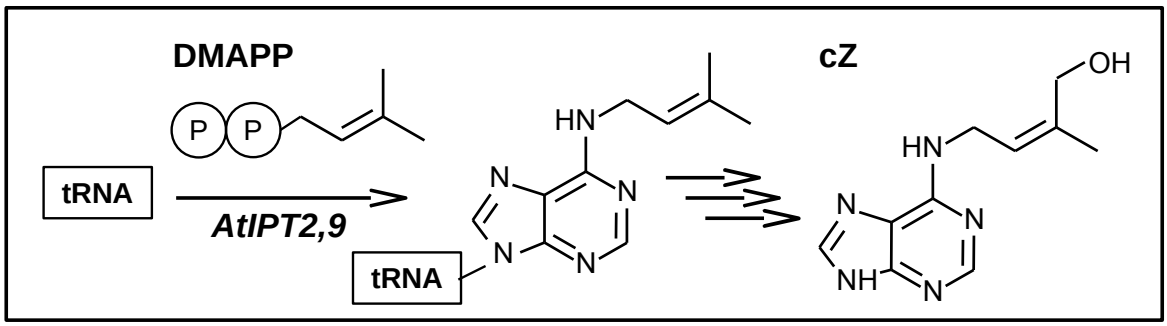

Figure 5: Biosynthesis of cis-zeatin (cZ). Arabidopsis isopentenyl transferases AtIPT2 and 9 transfer an isopentenyl moiety from dimethylallyl pyrophosphate (DMAPP) to adenine bases incorporated into tRNA. This reaction is followed by tRNA breakdown, which eventually yields cZ as a free base. The individual reactions are yet to be characterized. P: phosphate group.

defects. The results of studies on *Arabidopsis LOG* genes suggest that the LOG-mediated pathway plays a crucial role in cytokinin metabolism [76], [77]. Biosynthesis of tZ and iP is depicted in Figure 4.

Biosynthetic processes of other cytokinins than tZ and iP are less explored. A reductase converting tZ to DHZ was identified in common bean extracts [78], but no gene coding such an enzyme has been found yet. A cZ biosynthetic pathway from prenylated tRNA, mentioned above, is depicted in Figure 5. It was suggested that cZ may be produced via enzymatic interconversion of tZ. A putative enzyme catalysing such a reaction was partially purified [79]. However, a more recent study showed that the reaction observed *in vitro* was non-enzymatic and that the said interconversion is unlikely to occur in plants [80]. As for aromatic cytokinins, no model of their biosynthesis has been proposed yet.

2.3.2 Processes of Cytokinin Inactivation

There are two main ways of cytokinin free bases metabolic inactivation – oxidative cleavage at the N^6 atom, producing adenine and an oxidized form of the corresponding side chain, and conjugation of a cytokinin molecule with glucose. The oxidative cleavage is an irreversible process catalysed by cytokinin oxidases/dehydrogenases (CKXs). The CKX enzymatic activity was first observed in crude tobacco extracts, where radio-labelled iPR was converted to adenosine [81]. Similar activity was observed in lots of other types of plant material (reviewed in [82]). *ZmCKX1* gene from maize was the first *CKX* gene successfully cloned and expressed [83], [84]. Using bioinformatics tools, several other *CKX* genes were predicted, including seven genes in *Arabidopsis*, denoted *AtCKX1-7*. *AtCKX1-6* were shown to code functional enzymes [85], [86]. The CKXs had been originally believed to be copper-dependent amine oxidases, catalysing an electron transfer from the substrate to molecular oxygen and yielding hydrogen peroxide as a by-product. This view was supported by some apparent similarities between the CKXs and known copper-dependent oxidase, such as inhibition by cyanamide, post-translational glycosylation or formation of an imine intermediate (summarized in [82]). However, several later studies challenged this model. It was shown that CKXs are functional under anaerobic conditions [87]–[89] and that they contain a covalently-bound flavin cofactor, flavin adenine dinucleotide (FAD) [83], [84], [87]. The putative presence of copper in CKXs' active sites was disproved by both atomic absorption analysis and assays including a copper chelator [87]. When tested as potential substrates of an actual plant amine oxidase, cytokinins acted rather as inhibitors. It was also pointed out that amine oxidases

catalyse the oxidation of primary amines, whereas the cytokinin amino group at the N^6 position is a secondary one [90]. All the pieces of evidence considered, another model was proposed, wherein CKXs act as flavin-dependent dehydrogenases, transferring electrons from the substrate to FAD and ultimately to a final acceptor, which is yet to be determined [85], [88].

Cytokinin glycosylation, or conjugation with glucose, can occur at N^7 and N^9 atoms (*N*-glycosylation) of the adenine core or the *O* atom within the side chains of tZ, cZ and DHZ (*O*-glycosylation). The enzymes catalysing cytokinin glycosylation belong to a large family of UDP glycosyltransferases (UGTs), which recognize UDP glycosyl moieties as substrates [91]. In *Arabidopsis*, five *UGT* genes participate in cytokinin glycosylation. *UGT76C1* and *76C2* products are *N*-glycosyltransferases (each of the two enzymes catalyses both N^7- and N^9-glycosylation), whereas *UGT73C1*, *73C5* and *85A1* products are *O*-glycosyltransferases [92].

N-glycosylation is often characterized as irreversible; however, recent findings challenged this view [93]. Enzymes responsible for *N*-glycosylation, UGT76C1 and 76C2, were shown to accept tZ, cZ, DHZ, BAP, and kinetin as substrates [92]. Further studies revealed that *ugt76c1* and *76c2* mutations lead to decreased content of *N*-glycosylated cytokinins in plants and enhanced sensitivity to exogenously applied cytokinins. Conversely, *UGT76C1* and *76C2* over-expression leads to increased accumulation of *N*-glycosylated cytokinins in plants and decreased sensitivity toward exogenous cytokinins [94], [95].

Interestingly, the contents of cytokinins in forms of free bases in *ugt76c1* and *76c2* mutants remained similar to those in the wild type. Moreover, neither the *ugt76c1* mutant nor *UGT76C1* overexpressor displayed significant phenotype changes. These findings correspond with changes in expression patterns of genes participating in cytokinin metabolism; expression of *CKXs*, for instance, was elevated in *ugt* mutants and reduced in *UGT* overexpressors. Such a change in expression pattern compensates for the loss of *N*-glycosyltransferases action [93]–[95].

O-glycosylated cytokinins can be converted back to their active forms by a β-glucosidase, and they are hence considered to be a stable storage form of respective cytokinin species [40], [96].

The over-expression of *UGT85A1* yielded results analogous to those mentioned above – transgenic plants accumulated more cytokinin *O*-glucosides and they were less

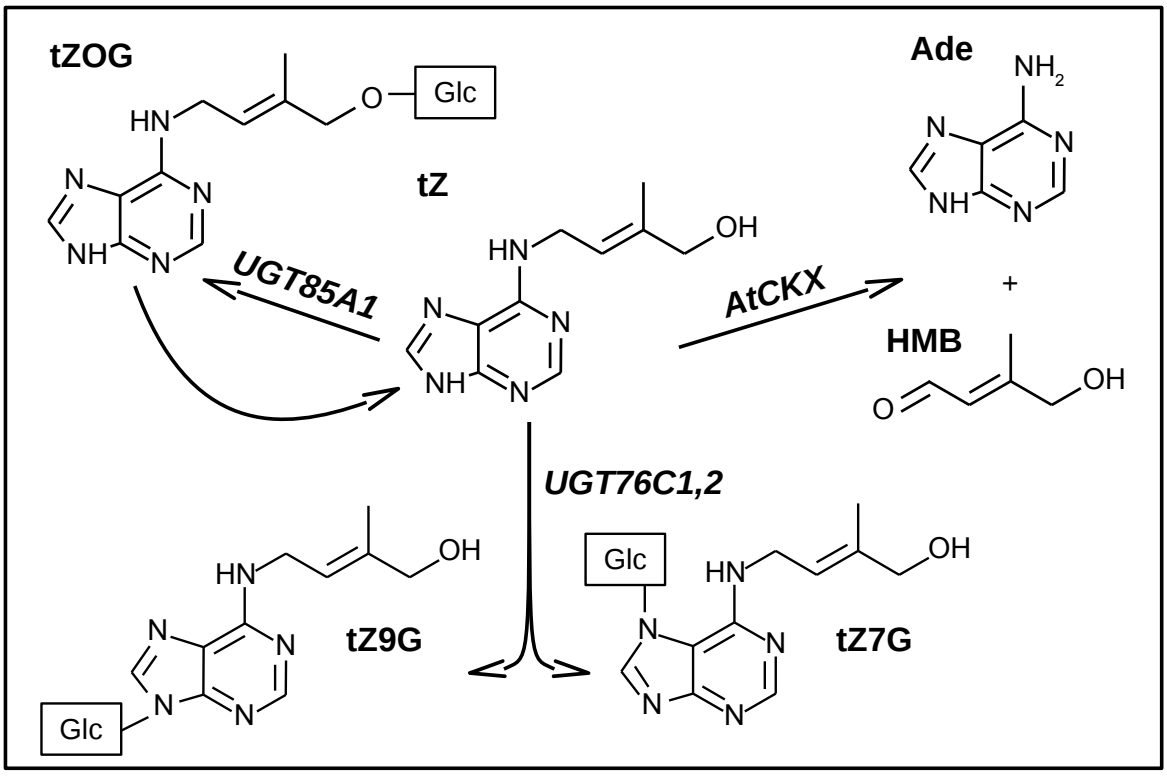

Figure 6: Cytokinin free bases conversion, shown on an example of trans-zeatin (tZ). Cytokinin oxidases/dehydrogenases (CKXs) catalyse irreversible tZ degradation, yielding adenine (Ade) and (E)-4-hydroxy-3-methyl-2-butenal (HMB). O-glycosylation of tZ is catalysed by UGT85A1 and produces trans-zeatin O-glucoside (tZOG). Conversion of tZOG back to tZ is catalysed by a β-glucosidase. N-glycosylation of tZ is catalyzed by UGT76C1 and 2. It occurs either on the N^7 or N^9 atom, yielding trans-zeatin N^7-glucoside (tZ7G) and trans-zeatin N^9-glucoside (tZ9G), respectively. Glc: glucose moiety. For complete structures, refer to Figure 3.

sensitive to exogenous cytokinins, in comparison to wild-type plants [97]. Processes of cytokinin inactivation are summarized in Figure 6.

2.4 Cytokinin Transport Mechanisms

2.4.1 Motivation of Cytokinin Transport Studies

The distribution of signal molecules, including the cytokinins, and their availability in organs, tissues and cell compartments greatly affects the processes modified by them. It follows that systems regulating their transport at all scales, ranging from membrane-associated transport proteins to vascular networks running throughout different tissues and organs, make up a powerful tool of plant growth and development control.

Concerning plant hormones, transport mechanisms have been so far studied mainly in relation to auxins. A detailed summary of auxin transport at the cellular level is given, for instance, in [98]. Apart from methods of molecular biology, biochemistry, analytical chemistry and so on, auxin transport has been also studied via mathematical modelling. First emerging in the '80s, various models of auxin transport have been proposed, focusing

mostly on its relation to vein formation [99]–[102] or phyllotaxis [103], [104]. A detailed review of auxin transport modelling is given in [105].

Extensive studies of plant transport mechanisms specific to cytokinins make up one of the contemporary plant hormone research topics. In the following parts, fundamental principles of membrane transport will be reviewed. For further reading on these topics, detailed textbooks dealing with the physical and chemical aspects of biological membranes and cellular transport, such as Lodish's "Molecular Cell Biology" [106] are recommended. Next, the individual types of membrane transport will be put in a context of cytokinins or other plant hormones and the most important works on cytokinin transport will be summarized as well.

2.4.2 Simple Diffusion

Diffusion is a spontaneous directional movement of mass from one compartment to another. It is driven by a concentration gradient of the substance in question and it causes the substance to move from the compartment where the concentration of the substance is greater to the one where it is smaller.

The physically chemical properties of diffusion were described by Adolf Fick [107], [108]. A theorem known as Fick's first law of diffusion describes proportionality between diffusion flux, $\vec{J}$ (i.e. the amount of mass passing through a unit of area per unit of time, in a dimension of mol×m^{-2}×s^{-1}), and the concentration gradient. Fick's first law states that:

$$\vec{J} = -D \nabla c \quad , \tag{1}$$

where c denotes the diffusing substance concentration (mol×L^{-1}) and D the diffusion coefficient (m^2×s^{-1}) The nabla symbol, ∇, denotes the gradient operator. The negative sign implies that the diffusion occurs in a direction against the concentration gradient, which itself is directed alongside increasing concentration, due to its mathematical definition.

Fick's second law describes how the concentration of the diffusing substance changes in time, i.e. the simple diffusion rate. It can be derived from Fick's first law as shown in [108]. Fick's second law states that:

$$\frac{\partial c}{\partial t} = D \nabla^2 c \quad , \tag{2}$$

where t denotes time (s).

Simple passive diffusion in terms of cellular transport requires passage of a given substance through the lipophilic inner environment of the phospholipid bilayer. Such a movement cannot occur if the molecules in question are too big or too polar (or, in extreme cases, charged) [106]. Uncharged cytokinin free bases and nucleotides can pass through cell membranes, as discussed in [109] and passive diffusion of cytokinins is often taken into account as a part of complex mathematical models. For instance, el-Showk et al. [110] examined cytokinin diffusion as one of the vascular patterning parameters. By varying the membrane permeability they explored possible effects of cytokinin spatial distribution on tissue development (which, however, turned out to be of little importance). Moore et al. [111] included cytokinin diffusion across cell membranes in a spatiotemporal model of major plant hormone species crosstalk during root development. In [112], it is stated that local production of cytokinins and subsequent longitudinal diffusion are key mechanisms of apical meristems maintenance.

Also, passive diffusion plays an important part in auxin polar transport. Being a weak acid (with pK_A of approximately 4.85), the most abundant auxin, IAA, is present in both dissociated and non-dissociated forms. Since the dissociated one bears a negative charge, the cell membrane is permeable for non-dissociated auxin only. Due to differences in pH values of cytoplasm (around 7.0) and apoplast (around 5.5), an equilibrium between both auxin forms is being made in the latter. Non-dissociated auxin molecules diffuse into the cell, where most of them dissociate, being thus prevented from returning to the apoplast. This simple model is known as the chemiosmotic hypothesis [98], [113]–[116]. The relation between the forms of weak electrolytes is in general given by Henderson-Hasselbalch equations, such as

$$pH = pK_A + \log\frac{[A^-]}{[HA]} \quad , \tag{3}$$

where pK_A denotes negative logarithm of the acidity constant, K_A; $[A^-]$ and $[HA]$ denote the concentrations of dissociated and non-dissociated forms of a weak electrolyte, respectively (mol×L^{-1}).

Considering that only the non-dissociated form is translocated via passive diffusion, Fick's first law (1) for weak electrolytes may be rewritten as:

$$\vec{J} = -\frac{D}{1+10^{pH-pK_A}} \times \nabla c \quad . \tag{4}$$

Therefore, the diffusion flux becomes a function of not only the concentration, but also of the pH, characterizing the environment, and the pK_A, characterizing acidic or basic

properties of the diffusing substance. To estimate the effect of these parameters, one can compare magnitudes of inward and outward diffusion flux on a cellular membrane driven by equally great concentration gradients. The inward flux, $\vec{J}_{inw}$, depends on outer parameters D^{out} and pH^{out}, while the outward flux, $\vec{J}_{outw}$, depends on inner parameters D^{in} and pH^{in}. Applying equation (4), it follows that:

$$\frac{\left|\vec{J}_{inw}\right|}{\left|\vec{J}_{outw}\right|} = \frac{D^{out}}{D^{in}} \cdot \frac{1+10^{\left(pH^{in}-pK_A\right)}}{1+10^{\left(pH^{out}-pK_A\right)}} \quad ; \tag{5}$$

neglecting the differences between diffusion constants and considering previously mentioned values of pK_A and pHs relevant to IAA, one finds that the inward flux magnitude is about 26 times greater, which is qualitatively in accord with the chemiosmotic hypothesis.

Ethylene, which is both low-molecular and rather hydrophobic, readily diffuses through the membranes as well. Being gaseous under standard conditions, it is emitted by plants, most notably by ripening fruits, similarly to other gases such as oxygen or carbon dioxide. In [117], ethylene emission rate was measured in mango fruits to determine the fruit skin resistance to gas diffusion, a parameter stemming from an expansion of Fick's first law.

2.4.3 Facilitated Diffusion

Generally speaking, few kinds of molecules are eligible for passing through biological membranes in the means of passive diffusion. The others must be translocated with help of membrane-localized transporters. A canonical transporter is usually described as a membrane-bound protein folded in such a manner that its hydrophobic residues are exposed on the outer surface, interacting with the membrane, while the polar residues face inward, creating a funnel and enabling for translocation of molecules without passing through the hydrophobic environment of the biological membrane. The transporters are specific for a certain substance or a structurally relevant group of molecules. This is achieved by a specific arrangement of polar groups throughout the inner surface so that their distances mimic the diameters of primary solvation envelopes of translocated molecules [106].

In the case of the facilitated diffusion, another type of membrane transport mechanism, the presence of membrane transporters doesn't affect the thermodynamic properties of the transport. In other words, the transport is still driven by the concentration

gradient, it is realized against the direction of this gradient and it occurs until the equilibrium of chemical gradient is achieved. The transporters, however, do greatly affect the transport kinetics.

Thanks to many similarities between membrane carriers and enzymes, facilitated diffusion kinetics can be described using the well-known Michaelis-Menten equation [118], [119], which states that:

$$v = \frac{V_{max}[S]}{K_M + [S]} \quad , \tag{6}$$

where v denotes initial reaction, or in our case transport rate (mol×L^{-1}×s^{-1}), V_{max} its maximal (or limit) value (mol×L^{-1}×s^{-1}), $[S]$ the substrate concentration (mol×L^{-1}), and K_M a constant related to the transporter affinity toward the substrate (mol×L^{-1}) – an analogue of Michaelis constant used in enzyme kinetics.

Apart from substrates, inhibitors bind to transporters as well. In the case of competitive inhibition, the substrate and the inhibitor act as rivals, competing for free binding sites provided by the transporter. Therefore, the transporter affinity toward the substrate is reduced. Equation (6) expands into:

$$v = \frac{V_{max}[S]}{\left(1 + \frac{[I]}{K_I}\right)K_M + [S]} \quad , \tag{7}$$

where $[I]$ denotes the inhibitor concentration (mol×L^{-1}) and K_I the inhibition constant (mol×L^{-1}), which characterizes the transporter's affinity toward the inhibitor.

2.4.4 Active Transport and ATP-Binding Cassettes in Plants

Unlike both types of diffusion discussed above, active transport of a substance occurs in the sense of its concentration gradient. It means that the substrate concentration gradient becomes greater as the transport proceeds. Such a process is highly endergonic and therefore it has to be coupled with an exergonic one.

A widely-employed exergonic process coupled to active transport is the hydrolysis of adenosine triphosphate (ATP), a ubiquitous macroergic molecule. Such a coupling is called primary active transport. Both ATP hydrolysis and substrate translocation is mediated by a single membrane-bound transporter – a member of ATP-binding cassette (ABC) family. It is safe to say that the ABC family plays a pivotal role in most living organisms, given a large number of its members and the fact that ABC transporters can be found throughout all organism kingdoms, both prokaryotic and eukaryotic [120], [121].

Canonical ABC transporters are composed of four functional domains; two nucleotide-binding domains (NBDs) are responsible for both binding and hydrolysis of ATP, whereas the other two, known as transmembrane domains (TMDs), keep the transporter anchored in the biological membrane and make up a pathway for a substrate being translocated. Based on their membrane topology, ABC transporters are classified as importers and exporters. Importers translocate the substrate to the same size of the membrane where their NBDs are to be found, whereas exporters translocate the substrate to the other side. Concerning eukaryotes, ABC importers have been found only in plants so far, while the exporters are present within all kingdoms. Prokaryotes have both ABC exporters and importers as well [122], [123].

There are over 130 genes coding the ABC transporters in the genome of *Arabidopsis thaliana*, or mouse-ear cress. These genes have been organized into eight subfamilies, denoted by letters A-G and I. This kind of classification is based on phylogenetic relations among individual genes and the domain organization of their products. Subfamilies *AtABCA-G* were defined according to a similar classification that had been done for human ABC transporters. They bear certain resemblances to their human counterparts and they code either a half-size or a full-size transporter. Members of the *AtABCI* subfamily, on the other hand, have no analogues in the human genome and their products consist of a single domain only. In this regard, they are similar to prokaryotic ABC transporters [122], [124], [125].

Plant ABC transporters are commonly associated with detoxification of xenobiotic compounds and distribution of many plant metabolites, including phytohormones [122]. Genes *AtABCB1, 4* and *19* are involved in auxin transport [126]–[129] and to interact with other auxin transporters, such as those of the *PIN-FORMED* (*PIN*) family [130]. Bailly et al. used methods of homologous modelling to predict crystal structures of *AtABCB1, 4*, *14* and *19*, that were subsequently analysed in terms of architecture, putative binding sites and substrate specificity [131]. Two members of another *ABC* subfamily, *AtABCG36* and *37* are involved in the transport of auxin precursors [132], [133]. As for other plant hormone types, genes *AtABCG25* [134] and *40* [135] were identified to code cellular exporter and importer of abscisic acid, respectively.

Additionally, the product of *AtABCG22* is suggested to play a role in abscisic acid transport as well, given that this hormone, among others, regulates induces stomatal closure, preventing water losses, and that the *atabcg22* mutant displayed a lower level of drought tolerance, compared to the wild type. However, direct evidence that abscisic acid

is a substrate of AtABCG22 is yet to be presented [136]. The share of the *ABC* gene family on cytokinin transport will be discussed separately in 2.4.5.

While primary active transport is bound to ATP hydrolysis and the action of ABC transporters, secondary active transport consists in coupling the endergonic translocation of one substrate with an exergonic translocation of another one. It follows that a carrier capable of transporting more than one substrate is required. Such a process is called cotransport and it can be further classified as symport if both substrates are translocated in the same direction, or antiport if translocation of the two substrates occurs in opposite directions. It should be noted that these cotransporters can be involved in all carrier-mediated transport processes, not only in the secondary active ones.

2.4.5 Major Families of Cytokinin Membrane Transporters

Given their general structural properties (see 2.2.1), the cytokinins are readily substrates of two major purine transporter families, namely of equilibrative nucleoside transporters (ENTs) and purine permeases (PUPs). Most of these carriers are non-specific toward cytokinins, which means that they transport an array of structurally related substrates (in this case, various derivatives of purines in some form), rather than a single chemical compound [137].

ENTs are present throughout all eukaryotic kingdoms. While mammalian ENTs are typical facilitated diffusion transporters, independent on concentration gradients of other substances [138], their plant counterparts mediate substrate-proton transport, which requires the presence of proton concentration gradient on the membrane [139]. As the name suggests, ENTs transport purines in form of nucleosides. They may be further classified as equilibrium-sensitive or insensitive, based on whether they are inhibited by nitrobenzylmercaptopurine ribonucleoside, a synthetic nucleoside analogue, or not, respectively [138]–[141]. Human ENTs have been profoundly studied as potential drug targets in various therapeutic applications [142], [143].

The first plant *ENT* gene, *AtENT1*, was found as a homologue of human *ENTs*. It was shown that such a gene is indeed expressed in *Arabidopsis* and that the corresponding product is localized to the plasma membrane [141]. Subsequent biochemical assays confirmed that *AtENT1* encodes a nucleoside transporter. When expressed in a yeast cell culture, AtENT1 transported radio-labelled adenosine according to Michaelis-Menten kinetics (see 2.4.3). In competition studies, AtENT1 displayed a relatively wide substrate specificity. The adenosine uptake was inhibited by other common nucleosides, with an

exception of uridine, and corresponding 2'-deoxynucleosides. Nucleotides, free bases and cytokinin nucleosides did not affect adenosine uptake, however [141], [144].

To show whether AtENT1 mediates facilitated diffusion or proton-dependent transport, it was tested in terms of sensitivity to carbonyl cyanide *m*-chlorophenyl-hydrazone (CCCP), a protonophore uncoupling the proton gradient. CCCP completely halted adenosine uptake, which revealed that nucleosides were symported with protons [145].

Following the studies on AtENT1, seven more *AtENT* genes (*AtENT2-8*) were expressed and characterized [146], [147]. It was shown that both *AtENT3* and *8* participate in iPR transport. Respective mutations in these two genes reduced sensitivity to iPR but not to iP. Conversely, iPR sensitivity was enhanced in plants over-expressing *AtENT8*. Additionally, hypocotyl explants bearing *atent3* and *atent8* mutations were limited in their ability to accumulate iPR. In the case of *atent3*, tZR uptake was lowered as well [144]. Similar results were obtained for *AtENT6* and *7*; in yeast cell cultures expressing these genes, adenosine uptake and its inhibition by iPR were observed [148]. Apart from *Arabidopsis*, four *ENT* genes were also found in rice (*Oryza sativa*). They are denoted *OsENT1-4*. When expressed in a yeast cell culture, *OsENT2* displayed affinity toward iPR [149].

Unlike *ENT*s, the *PUP* gene family is unique to vascular plants and it is involved in the transport of purines in a form of free bases [150]–[152]. In total, 21 *PUP* genes have been identified [137]. The first *PUP* gene, *AtPUP1*, was found via functional complementation of a mutant yeast cell culture deficient in adenine uptake. Further biochemical characterization revealed that AtPUP1, expressed in yeast cells, transports a wide spectrum of substrates, including cytosine, hypoxanthine (but not uracil nor thymine), purine alkaloids, such as nicotine or caffeine, and cytokinins. Affinity toward some nucleosides was observed as well, albeit significantly weaker than affinity toward corresponding free bases. AtPUP1 is also sensitive to protonophores and H^+-ATPase inhibitors, indicating proton-coupled secondary active transport, similarly to ENTs [150], [153]. Similar results were obtained for AtPUP2. On the other hand, AtPUP3 showed no transport activity in yeast heterologous expression systems, neither did it complement the adenine-uptake deficiency in mutant yeast cell cultures [153]. In [109], the authors demonstrated that there is a CCCP-sensitive cytokinin transport system in *Arabidopsis* seedlings. Suggesting that PUPs are involved, they further studied expression patterns of twelve *AtPUP* genes. *AtPUP11*, *14* and *18* were highly expressed in all plant tissues. In

roots, a significant expression level of *AtPUP4*, a close homologue of *AtPUP1* and *2*, was detected as well.

Twelve *PUP* genes, denoted *OsPUP1-12*, were identified in the rice genome. The *ospup7* mutation caused several phenotype changes, such as plant height increase, larger seeds or delayed flowering. Cytokinin measurements showed higher iP and iPR content in *ospup7* immature spikelets in comparison to the wild type, while tZ-type cytokinin level remained unaffected. It was also demonstrated that OsPUP7 can transport caffeine [154]. In tobacco (*Nicotiana tabacum*), one *PUP* gene, *NtPUP1*, was identified as well. It was characterized as a specific transporter of nicotine, whose expression is coordinately regulated with genes participating on nicotine biosynthesis. No role of NtPUP1 in cytokinin transport was observed [152].

Two cytokinin transporters are directly linked to the cytokinin signalization, so far. They are AtPUP14 and AtABCG14 (see 2.4.4 for more information on ABC transporters). The role of AtPUP14 was first described by Zürcher et al. in [155]. To assess factors affecting cytokinin action, the authors expressed a synthetic cytokinin reporter [156] within *Arabidopsis* heart-stage embryos. They detected no response to cytokinin signal in prospective cotyledons, even though all the components of cytokinin signalization cascade were present and functional. Therefore, they hypothesized that interactions of cytokinins and their receptors may be mediated by membrane transporters which regulate cytokinin availability to the receptors on one side of the membrane. Mapping expression of *PUP* genes, they found out that AtPUP14 is localized to those sites where cytokinin response was inhibited. When expressed in heterologous systems, AtPUP14 was shown to enhance tZ uptake. The uptake was inhibited by iP and BA but not by tZ riboside, auxins or allantoin, suggesting that AtPUP14 has an affinity toward cytokinin free bases only [155].

The role of the other transporter, AtABCG14, in cytokinin signalization was independently reported by two research groups. Ko et al. [157] were searching for genes involved in cytokinin long-distance transport. *AtABCG14*, which is coexpressed with genes of cytokinin biosynthesis in root phloem companion cells and whose expression is inducible by cytokinins, occurred to them as a good candidate. The *atabcg14* mutants displayed a phenotype reminding of mutations of cytokinin biosynthesis genes. This phenotype could be recovered with the exogenous application of tZ. Other experiments revealed that *atabcg14* shoots contained reduced concentrations of tZ-type cytokinins, whereas in roots the tZ-type cytokinin concentrations were elevated. *atabcg14* seedlings were impaired in root-to-shoot translocation of radio-labelled tZ. This was further

supported by showing that grafting an *atabcg14* shoot on a wild-type root recovers the mutant shoot phenotype while grafting a wild-type shoot on an *atabcg14* root caused the shoot to adopt a phenotype of the *abcg14* mutant. Therefore, the authors proposed that AtABCG14 exports tZ-type cytokinins to phloem in roots. However, they weren't able to provide direct evidence of *AtABCG14* transport function in a heterologous yeast expression system. Since *AtABCG14* is a half-size ABC transporter, it likely forms a functional heterodimer. It was shown in another paper, that AtABCG11/14 heterodimer is readily formed (unlike AtABCG14/14 homodimer) in *Arabidopsis* vascular system [158]. Ko et al. found the formation of such a heterodimer in roots unlikely, though [157].

In the same year, Zhang et al. [159] were systematically characterizing the members of *AtABCG14* gene subfamily. They found out that *atabcg14* displays a distinct phenotype, similar to that described in [157]. To map cytokinin distribution *in planta*, the authors introduced expressed a fusion gene, combining a gene responding to cytokinin signalization and a reporter gene, in both wild-type and *atabcg14* plants. In mutant plants, cytokinins were exclusively found in roots, whereas in wild-type plants, most cytokinins were found in the shoot. In both works, authors concluded that AtABCG14 serves as a tZ-type cytokinin exporter localized in roots, playing a crucial role in cytokinin root-to-shoot translocation [157], [159].

2.5 Analytical Methods in Cytokinin Research – Development and Application

2.5.1 Methods of Cytokinin Extraction

Studying cytokinins *in planta* is limited by their naturally low abundance, a huge amount of other compounds occurring in plants and enzymatic activity leading to various metabolic conversions. Thus, to study cytokinins using common analytical methods, one has to begin with their extraction from plant material [160].

Once the plant material has been homogenized (by grinding or sonification, for instance), cytokinins may be extracted into a liquid phase. Usually, mixtures of simple organic or aqueous solvents, such as methanol, ethanol, chloroform, ethyl acetate, formic acid or perchloric acid are used [161]–[164].

Enzymatic cleavage of phosphoester bonds present in cytokinin nucleotides has a negative impact on extraction yields. Because of this, extraction agents capable of inactivating plant phosphatases are preferred. In 1964, Roderick Bieleski developed the

MCF-7 solution consisting of 12:5:1:2 (v/v/v/v) mixture of methanol, chloroform, formic acid and water [165]. It was reported that the Bieleski solution reduces AMP hydrolysis in soya bean callus tissue and [14C]-labelled benzyl adenine nucleotide, though direct evidence of plant phosphatases inhibition during extraction is yet to be shown [160], [162].

However, the chloroform present in the Bieleski solution was reported to cause unwanted extraction of lipophilic substances, subsequently interfering with further purification. Moreover, when comparing the plant phosphatases inhibition by Bieleski solution with effects of either 80% (v/v) ethanol [162] or cold perchloric acid [164], similar results with little difference were obtained.

To see whether the chloroform can be omitted without hindering the extraction capacity of Bieleski solution, three types of extraction and isolation experiments using deuterated cytokinins were conducted [160]. Accordingly, three different extraction solutions were tested, namely 80% (v/v) methanol, Bieleski MCF-7 and modified Bieleski solution, 15:1:4 (v/v/v) mixture of methanol, formic acid, and water. The quality of each extraction was expressed in terms of relative internal standard response (RISR), i.e. the ratio between the areas under curves representing the signals from plant material spiked with deuterated cytokinins and pure deuterated standard. Using the modified Bieleski solution yielded the highest RISR percentages while using both MCF-7 and modified Bieleski solution resulted in lower dephosphorylation of cytokinin nucleotides [160]. Thus, it was affirmed that leaving out the chloroform of Bieleski MCF-7, which had been already done earlier [162], [166], is not only an alternative approach but even an improvement, given the higher RISR percentages and safer handling.

2.5.2 Methods of Cytokinin Purification

The cytokinin extraction procedures described in previous part lead to extracts which are complex mixtures of various substances. Therefore, the next step in cytokinin analysis is the extract purification. Once again, several methods regarding the purification process have been described.

Due to the functional groups capable of acting as Brønsted acids or bases, cytokinins may exist in ionized forms. In adenine, the imino nitrogen atom has pK_A about 4, which makes it positively charged in an acidic environment. The 2’-hydroxyl groups of purine nucleosides and nucleotides act as acids with pK_A over 12. There is also a phosphate group in cytokinin nucleotides, a strong acid that can be double deprotonated with pK_A values of about 1 and 6 [167]–[170].

Thanks to those acid-base functional groups, ion-exchange (IEX) methods may be employed in purification protocols. Anion-exchange can be used to separate cytokinin nucleotides from other forms. It is usually performed using columns filled with DEAE-cellulose or DEAE-Sephadex. DEAE, diethylaminoethyl, bears a positive charge in neutral pH. When cytokinin mixture is applied, the nucleotides are retained, whereas the free bases, ribosides and glucosides are not [166], [169].

Another important chemical property of cytokinins, related to their purification, is their hydrophobicity. Thanks to their N^6-side chain, they are more hydrophobic than corresponding forms of adenine. Individual cytokinin species also differ in their hydrophobicity due to different compositions of the N^6-side chains or presence of ribosyl and phosphoryl components [169].

Based on those differences in hydrophobicity, the cytokinin purification can be performed with the solid phase extraction (SPE) as well. Such procedures are typically conducted on a C18 bound silica stationary phases. These act as reversed phases, which means that hydrophobic compounds are retained on such a column, whereas polar ones are eluted with the mobile phase. Cytokinin species bound to the stationary phase may be washed out afterwards, using agents such as methanol or ethanol [166], [169], [171], [172]. It was reported that cytokinin nucleotides could not be recovered and that their hydrolysis to nucleosides before the purification was required [171].

To profit from both IEX and SPE, and to overcome their disadvantages to some degree, columns with properties of both reversed-phase and cation-exchange phase (so-called mixed-mode-SPE) are manufactured. Dobrev and Kamínek developed a mixed-mode-SPE-based method for separation of the cytokinin fraction from acidic phytohormones, mainly auxins and abscisic acid, followed by separation of cytokinin nucleotides from the other forms [169]. Later, a similar method combining the properties of reversed-phase and anion-exchange was developed by Dobrev et al. to purify auxins and abscisic acid [173].

The mixed-mode-SPE was also compared to C18 silica bound reversed-phase in tandem with anion-exchange columns, which is described for instance in [166]. Both protocols gave similar yields of most tested cytokinins, except for tZR and tZRMP, where the yields obtained with the former method were significantly higher. Also, the aforementioned RISR parameter provided by the mixed-mode-SPE was higher for all tested substances [160].

The immunoaffinity chromatography (IAC) methods can be used to achieve a high degree of purification. They are based on specific interactions between an antibody and its antigen, in our case a cytokinin. The antibodies against cytokinins are usually prepared by conjugating certain cytokinin species to bovine serum proteins, such as albumin or γ-globulins and subsequent immunization of laboratory animals [166], [174]–[177].

It was shown that monoclonal antibodies derived against iP may show a high degree of cross-reactivity with other cytokinins, while no cross-reactivity with non-cytokinin species, such as adenosine, was observed [177]. The cross-reactivity was sufficient to purify a cytokinin fraction via IAC using a single monoclonal antibody line. In case that the cross-reactivity of a derived monoclonal antibody line is not sufficient, the IAC may be performed using a mix of several such antibodies, as discussed in [174]. Monoclonal antibodies (or their mixtures) don't bind *O*-glycosylated forms of cytokinins, though. To isolate these species as well, one can enzymatically hydrolyse the eluted glucosides with β-glucosidase and repeat the purification protocol [178]–[180]. Interestingly, authors who had opted for running IAC using polyclonal antibodies didn't report any need for the glucosidase [181]–[183]. In [182], it is explicitly said that the polyclonal antibodies used in that study, raised against various cytokinin nucleosides, were able to bind cytokinin N^7-glucosides as well. Regardless of the clonality of antibodies, the cytokinin nucleotides were enzymatically dephosphorylated before further analysis in most aforementioned studies [179]–[183].

Concerning protein IAC, the oriented immobilization of antibodies generally enhances the binding capacity of the immunosorbent [184], [185]. To test whether the same can be said about low-molecular cytokinins, the authors in [174] compared various parameters of random and oriented antibody immobilization. No crucial differences were observed, except for the specific column capacity (i.e. column capacity per mass of immobilized antibody), being somewhat higher for oriented immobilization. This parameter, however, reflected not only binding efficiency but also protein loading, which was different in each case.

2.5.3 Methods of Identification and Quantification of Cytokinins

Once the cytokinin fraction has been sufficiently purified, individual cytokinins can be identified and quantified. The methods commonly used in this step are gas or liquid chromatography in tandem with mass spectroscopy (GC/MS and LC/MS, respectively) and

immunological methods, such as enzyme-linked immunosorbent assay (ELISA) or radioimmunoassay (RIA).

In case of the GC/MS, the analytes present in the sample must be converted to apt derivatives. This step seeks to improve the volatility of analytes, so that they can be carried by the gaseous mobile phase, as well as to stabilize some compounds and to facilitate subsequent chromatographic separation and ionization, necessary for the MS analysis [186], [187]. Several methods of cytokinin derivatization, or the introduction of a suitable functional moiety to cytokinin molecules, have been described [186]–[190].

A method based on vapour phase extraction (VPE) was designed for fast and efficient preparation of plant material before the GC/MS [186], [191]–[193]. Acidic compounds present in the homogenized material are converted to their corresponding methyl esters either via acid-catalysed reaction with methanol [191] or by using trimethylsilyldiazomethan, a stable and safe agent [192], [193]. Details on the latter esterification technique are given in [194]. The VPE method hasn’t been used for cytokinin analysis but it has proved to be useful in profiling acidic phytohormones, such as IAA, jasmonates or salicylic acid, as well as other volatile metabolites or toxins. One of its advantages lies in avoiding a multiple-step purification process, which would be otherwise similar to those described in 2.5.2 [191]–[193].

Alongside the derivatization technique, a choice of an ionization method for the MS analysis has to be made as well. In combination with the GC, the phytohormone samples are usually subjects of either electron ionization (EI; also called electron impact ionization) or chemical ionization (CI). The former consists of thermionic emission of electrons from a heated filament. These electrons are accelerated by an external electric field. Eventually, the accelerated electrons collide with the analyte molecules producing a relatively large number of ionic fragments, among which the molecular ion (denoted as M^+), possessing the same molecular mass as the original analyte, may be present. The CI, on the other hand, doesn't involve the direct collision of the thermionic electrons with the analyte, but rather with a reactant gas. Reactant ions, produced in this way, then serve as an agent ionizing the analyte itself. Compared to the EI, less energy is involved and fewer fragments are formed [186], [195]–[197].

Unlike the GC, the LC analyses substances in a solution, thus no derivatization is needed. Methods employing the LC/MS technique were used, for instance, in cytokinins [180] and brassinosteroids profiling [198]. As mentioned in [186], employing the LC/MS for phytohormone profiling may require an additional analytical method to increase the

overall selectivity or to confirm the previously obtained results, such as the ultraviolet spectroscopy in [198] or immunochemical methods in [180]. Dobrev et al. [173] used a method of two-dimensional high-performance liquid chromatography (HPLC) [199] as a form of sample purification, followed by either a spectroscopic measurement or the GC/MS.

If HPLC coupled with a tandem mass spectrometer is used (LC/MS/MS or HPLC/MS/MS), much higher selectivity is achieved. MS/MS enables to select precursor ions, produced in the first MS step, and their further fragmentation, yielding so-called product ions, whose analysis is the final output of this method [186]. The HPLC/MS/MS has been used in various profiling studies, often yielding information about a wide range of plant hormones [160], [186], [196], [200]–[204]. It is noted in [160] that the HPLC/MS/MS is a popular method of cytokinin analysis because of its wide range and its capacity to detect and determine the *O*-glycosylated cytokinins.

Apart from the chromatography, immunochemical methods have been used as other cytokinin profiling techniques. Similarly to the IAC described in 2.5.2, they are based on specific interactions between an antibody and its antigen (the compound of interest). The ELISA consists of building a so-called “sandwich complex” composing of an antigen, a primary antibody, raised against this antigen, a secondary antibody, raised against the primary one, and an enzyme. One of these components, depending on the specific protocol, is immobilized on a solid phase. When such a structure is formed, a substrate of the said enzyme is added and subsequently converted to a detectable product, whose amount is proportional to the amount of the antigen present in the sample. Several ELISA arrangements are used for plant hormones analysis [196], [205], [206]. Similarly to the IAC, *O*-glycosylated cytokinins require hydrolysis before being determined with the ELISA [207].

The RIA, developed as a method of insulin determination by Rosalyn Yalow and Solomon Berson [208], [209], makes use of a competition between a radioactively labelled antigen, provided in a defined amount, and its non-labelled counterpart, originating from the sample, over binding sites of the primary antibodies. Once an equilibrium between all the antigen forms is reached, secondary antibodies are added and complexes similar to those described for ELISA are formed. Next, they are separated from the liquid phase, which contains unbound reaction components, via centrifugation as the pellet. The analyte determination is realized through radioactivity measurement. Such a signal is inversely proportional to the amount of analyte in the sample [210]. The RIA is used as a method of

cytokinin profiling, for instance, in [176], [211], [212], and further discussed in reviews such as [196], [213]. It is stated in [213] that the RIA's popularity has declined in favour of the ELISA, due to special requirements stemming from the work with radioactive material.

3 Aims of the Thesis

Properties of cytokinin membrane transport were previously studied in my bachelor thesis [214] and, together with the cytokinin metabolism in tobacco BY-2 cells, also in Petr Klíma's PhD thesis [215]. In both cases, we were interested in cytokinins in forms of free bases and nucleosides, in particular. It was found out that both these species are readily accumulated in BY-2 cells. Transgenic expression of *AtPUP14* in BY-2 cells further enhanced the accumulation of both free bases and nucleosides, while no significant change in cytokinin accumulation was observed after transgenic expression of *AtABCG14* in BY-2 cells. Some competition was observed while accumulating multiple cytokinin species of the same type but there seemed to be no such competition when a free base was accumulated with a nucleotide. It was suggested that plant cells possess distinctive kinds of mechanism to transport different types of cytokinins.

Based on these results, this thesis aims to:

1. characterize the membrane transport of radio-labelled cytokinin free bases and N^9-glucosides in tobacco BY-2 cell suspension cultures
2. study the effects of *AtPUP14* transgenic expression in BY-2 cells on membrane transport of radio-labelled cytokinins in such cells
3. study the kinetic properties of membrane transport for different cytokinin types in BY-2 cells to characterize their mutual competition
4. describe metabolic processes of cytokinin in transgenic *Arabidopsis thaliana* plants having genes for specific cytokinin transporters deleted
5. study possible effects of deletions mentioned above on *de novo* organogenesis in *Arabidopsis*.

4 Material and Methods

4.1 Plant Material

4.1.1 Transgenic *Arabidopsis thaliana* seeds

Transgenic seeds of *Arabidopsis thaliana* muted in *AtENT*, *AtPUP*, or *AtABCG* genes were purchased from Eurasian Arabidopsis Stock Centre (NASC). A list of purchased transgenic lines is given in Table 1. Other *Arabidopsis* lines included an At*UGT76C2*-overexpressing line and a wild type line. Both these lines were provided by the Institute of Experimental Botany of the Czech Academy of Sciences.

Table 1: A list of transgenic Arabidopsis lines purchased from Eurasian Arabidopsis Stock Centre (NASC). Each line is identified with a NASC accession code (the second column). Furthermore, two more references are provided for each mutation, namely the UniProt accession number (the third column) and the Arabidopsis Genome Initiative gene code (AGI; the fourth column), denoting the mutation locus within the Arabidopsis thaliana genome.

Mutation	NASC Accession Number	UniProt Accession Number	AGI Gene Code
atpup1	N661084	Q9FZ96	At1g28230
atpup14	N671081	Q9FXH5	At1g19770
atent1	N667333	Q8VXY7	At1g70330
	N604866		
atent3	N631585	Q9M0Y3	At4g05120
	N3729		
atent7	N667574	Q944N8	At1g61630
atent8	N639438	Q944P0	At1g02630
atabcg14	N1002995	Q9C6W5	At1g31770

4.1.2 Tobacco BY-2 Cell Lines

Tobacco (*Nicotiana tabacum*) BY-2 cell lines [216] were suspended in a liquid medium consisting of 4.33 g×L^{-1} Murashige-Skoog salt mixture [217], 1 g×L^{-1} thiamine, 200 µg×L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 200 mg×L^{-1} monopotassium phosphate, 100 mg×L^{-1} *myo*-inositol, and 30 g×L^{-1} sucrose, with pH adjusted to 5.8, further referred to as the BY-2 medium. Transgenic BY-2 lines were inducible by estradiol (the final estradiol concentration was 900 nmol×L^{-1}). All the BY-2 lines were derived at the Institute of Experimental Botany of the Czech Academy of Sciences.

4.2 Chemicals

4.2.1 Radio-Labelled Cytokinin Tracers

Cytokinins used as radio-tracers were provided by Izotope Laboratory of the Institute of Experimental Botany of the Czech Academy of Sciences. The tracers were labelled by ^{3}H nuclide. In the text, they are denoted as [3H]-tZ, [3H]-iP, [3H]-tZ9G, [3H]-iP9G, and [3H]-cZ9G. They were kept at -80 °C.

4.2.2 Media and Solutions

Compositions of all media and solutions are summarized in Table 2.

Table 2: Summary of media and solutions used.

Solution/Medium	Composition
BY-2 medium (liquid)	4.33 g×L^{-1} Murashige-Skoog salt mixture, 1 mg×L^{-1} thiamine, 200 µg×L^{-1} 2,4-dichlorophenic acid (2,4-D), 200 mg×L^{-1} monopotassium phosphate, 100 mg×L *myo*-inositol, 30 g×L sucrose; pH = 5.8 (adjusted with potassium hydroxide)
K0 medium (solid)	4.33 g×L^{-1} Murashige-Skoog salt mixture including Gamborg B5 vitamins [218], 10 g×L^{-1} sucrose, 3 g ×L^{-1} Phytagel; pH = 5.7 (adjusted with potassium hydroxide)
Cytokinin-containing media (solid)	4.33 g×L^{-1} Murashige-Skoog salt mixture including Gamborg B5 vitamins [218], 10 g×L^{-1} sucrose, 1 mg×L biotin, 3 g×L^{-1} Phytagel, 100 µg×L^{-1} 1-naphtylacetic acid, cytokinins according to Table 4; pH = 5.7 (adjusted with potassium hydroxide)
Uptake buffer	20 mmol×L^{-1} 2(*N*-morpholino-)ethanesulfonic acid (MES), 10 mmol×L^{-1} sucrose, 0.5 mmol×L^{-1} calcium sulphate; pH = 5.7 (adjusted with potassium hydroxide)
MS/2 medium (liquid)	2.17 g×L^{-1} Murashige-Skoog salt mixture; pH = 5.7 (adjusted with potassium hydroxide)
MS/2 medium (solid)	2.17 g×L^{-1} Murashige-Skoog salt mixture, 1.2 g×L^{-1} agar; pH between 5.6 and 5.9 (adjusted with potassium hydroxide)
Modified Bieleski solution	15:1:4 (v/v/v) mixture of methanol, formic acid, and water
Elute B	0.35 mol×L^{-1} ammonium hydroxide in 70% (v/v) methanol
HPLC mobile phase A	400 mmol×L^{-1} ammonium acetate; pH = 3.8 (adjusted with acetic acid)
HPLC mobile phase B	5% (v/v) methanol
HPLC mobile phase C	1:1 (v/v) mixture of acetonitrile and methanol

4.2.3 Other Chemicals

For radioactivity measurements, liquid scintillation cocktail Flo-Scint III (PerkinElmer, USA) was used.

4.3 Equipment

Equipment used in this work is summarized in Table 3.

Table 3: Summary of equipment used.

Equipment	Manufacturer (Country)
Centrifuge 5430 R (88 mm rotor radius)	Eppendorf AG (Germany)
Extraction cartridges Oasis MCX 1cc	Waters (USA)
HPLC flow-through radioactivity detector Ramona 2000	Raytest GmbH (Germany)
Inversion microscope Axiovert 40 C	Carl Zeiss AG (Germany)
Liquid scintillation counter TRI-CARB 4910TR 110V	PerkinElmer (USA)
Mixer mill MM 301	Retsch GmbH (Germany)
Orbital shaker IS-971R (30 mm orbital diameter)	Jeio Tech (South Korea)
Orbital shaker KS 130 (4 mm orbital diameter)	IKA (USA)
Reverse-phase HPLC column Kinetex C18 (150 × 4.6 mm, 5 µm)	Phenomenex (USA)
Rotary vane pump VR 1,5/12	Lavat (Czechia)
Rotational vacuum concentrator Alpha	Martin Christ Gefriertrocknungsanlagen GmbH (Germany)
Ultra low freezer MDF-U700VX	PHCbi (Japan)
Vacuum manifold Visiprep SPE	Supelco (USA)

4.4 Methods

4.4.1 Tobacco BY-2 Cell Lines Treatment

The BY-2 cell lines were kept suspended in liquid BY-2 medium (see Table 2). To keep the BY-2 lines alive, inocula of respective cell suspensions were transferred into fresh BY-2 medium weekly. 1 mL of a cell suspension was an adequate amount to inoculate 30 mL of fresh BY-2 medium. The suspensions were cultivated on IS-971R orbital shaker (Jeio Tech) at 1 g (150 RPM) and 27 °C in the dark. The transgenic lines were cultivated in the presence of selection antibiotics (300 $mg \times L^{-1}$ Claforan and 60 $mg \times L^{-1}$ hygromycin B).

4.4.2 *Arabidopsis* Seeds Sterilization

To prevent contamination, *Arabidopsis* seeds were sterilized before further cultivation. This was achieved by successive two-minute treatment of the seeds with 70% (v/v) ethanol, ten-minute treatment with 10% (v/v) SAVO® containing a small amount of Tween, and triple washout of the seeds with sterilized deionized water.

4.4.3 *Arabidopsis* Hypocotyl Assays

The hypocotyl assay procedure is described in [219], [220]. Sterilized *Arabidopsis* seeds (see 4.4.2) were seeded on Petri dishes with a solid medium consisting of 4.33 $g \times L^{-1}$ Murashige-Skoog salt mixture including Gamborg B5 vitamins [218], 10 $g \times L^{-1}$ sucrose, and 3 $g \times L^{-1}$ Phytagel, with pH adjusted to 5.7, further referred to as K0, out of analogy with the media denotation introduced in [219]. Dishes with the medium were covered and sealed with paper tape to allow gas exchange. The seeds were kept overnight in the dark at -4 °C, then under the long-day conditions (altering 16-hour long light periods and 8-hour long dark periods) at 21 °C for one day and in the dark at 21 °C for five days.

Having emerged in the dark, the seven-day-old plants had long hypocotyls, typical for etiolated seedlings. These hypocotyls were aseptically isolated by cutting off the apical hook and roots and transferred to Petri dishes with a solid medium consisting of 4.33 $g \times L^{-1}$ Murashige-Skoog salt mixture including Gamborg B5 vitamins [218], 10 $g \times L^{-1}$ sucrose, 1 mg×L biotin, 3 $g \times L^{-1}$ Phytagel, 100 $\mu g \times L^{-1}$ 1-naphtylacetic (NAA) acid, and a variable concentration of a cytokinin, with pH adjusted to 5.7. The cytokinins and their concentrations used in hypocotyl assays are summarized in Table 4. The Petri dishes were sealed with paper tape and the hypocotyls were further cultivated for 21 days under the long-day conditions at 21 °C.

Table 4: Types of phytohormone-enriched media used in hypocotyl essays. Different types of media vary in cytokinin type and concentration. For future references, the media are labelled as shown in the first column. The labels follow a naming convention presented in [219].

Medium Label	Cytokinin Type	Cytokinin Concentration [$\mu g \times L^{-1}$]	Auxin (NAA) Concentration [$\mu g \times L^{-1}$]
K30	Kinetin	30	100
K100		100	
K300		300	
I300	iP	300	
Z300	tZ	300	

4.4.4 Metabolic Assays in *Arabidopsis* Plants

Sterilized *Arabidopsis* seeds (see 4.4.2) were seeded on rectangular Petri dishes with solid MS/2 medium consisting of 2.17 g×L^{-1} Murashige-Skoog salt mixture and 1.2 g×L^{-1} agar, with pH between 5.6 and 5.9. Dishes with the medium were covered and sealed with paper tape to allow gas exchange. The seeds were kept for two days in the dark at -4 °C, then under the long-day conditions (altering 16-hour long light periods and 8-hour long dark periods) at 21 °C for 14 days in the vertical position.

The 14-day long *Arabidopsis* plants were submerged in liquid MS/2 media, i.e. 2.17 g×L^{-1} Murashige-Skoog salt mixture with pH between 5.6 and 5.9, containing a radio-labelled tracer. The tracer concentration in liquid MS/2 media was 20 nmol×L^{-1}. The plants were treated in these media for 100 minutes. After the treatment, cytokinin fractions were isolated from the plant samples (see 4.4.5) and subjected to the HPLC analysis (see 4.4.6).

Alongside the cytokinin fractions obtained from *Arabidopsis* plants, the media containing the radio-labelled tracers were analysed too. From each medium, 100 µL samples were taken both before and after the plant treatment. From each sample, a 10 µL aliquot was taken for total radioactivity determination (see 4.4.8). The rest of each sample was subjected to the HPLC analysis (see 4.4.6).

4.4.5 Cytokinin Profiling in *Arabidopsis* Plants

The determination of cytokinin species and their respective concentration was based on the profiling method described in [169]. Frozen samples of plant material (of mass 10 – 1000 mg) were suspended in the modified Bieleski solution – a 15:1:4 (v/v/v) mixture of methanol, formic acid, and water. The suspensions were homogenized in MM 301 mixer mill (Retsch) at the frequency of 30 Hz for four minutes. Homogenized samples were placed into -20 °C for 60 minutes and then centrifuged in 5430 R centrifuge (Eppendorf) at 28,433×g (17,500 RPM).

After the centrifugation, the supernatants were put aside on ice and the pellets were resuspended in 500 µL of the modified Bieleski solution. The samples were once again homogenized and centrifuged as described above. After the second centrifugation, the supernatants were separated from the pellets again and mixed with the corresponding supernatant fraction taken before. The remaining pellets were thrown away and the liquid

samples were placed into -80 °C for 30 minutes and then evaporated on Alpha rotational vacuum concentrator (Christ) until approximately 200 µL of the liquid were remaining.

Oasis MCX 1cc extraction cartridges (Waters) were placed on Visiprep SPE vacuum manifold (Supelco). The cartridges were activated by being subsequently washed with 1 mL of methanol and 1 mL of 1 mol×L^{-1} formic acid. The liquid samples remnants were reconstituted in 500 µL of 1 mol×L^{-1} formic acid and applied to the activated cartridges. The sample containers were twice washed with 250 µL of 1 mol×L^{-1} formic acid and emptied to the extraction cartridges. To remove lipids and plant pigments from the samples, the cartridges were washed with 500 µL of 1 mol×L^{-1} formic acid and 250 µL of deionized water.

A fraction containing acid phytohormones (such as auxins and abscisic acid) was eluted by washing the cartridges with 250 µL of methanol three times. Eventually, the cytokinin fraction was eluted by washing the cartridges with so-called elute B, composing of 0.35 mol×L^{-1} ammonium hydroxide in 70% (v/v) methanol.

Once the desired fraction was isolated, it was evaporated to dryness and reconstituted in 50 µL of 15% (v/v) acetonitrile. The samples were put into -80 °C for 30 minutes and then centrifuged, as described above. The supernatant was transferred to vials compatible with a high-performance liquid chromatograph.

4.4.6 High-Performance Liquid Chromatography Analysis of Cytokinin Metabolites

High-performance liquid chromatography (HPLC) was used to identify and quantify radio-labelled cytokinin metabolites. The analysis was performed using Kinetex C18 column, 150 × 4.6 mm, 5 µm (Phenomenex). Mobile phase A was 400 mmol×L^{-1} ammonium acetate, with pH adjusted to 3.8 with acetic acid, mobile phase B was 5% (v/v) methanol, and mobile phase C was a 1:1 (v/v) mixture of acetonitrile and methanol. The flow rate was 0.6 mL×min^{-1} with the following linear gradients of mobile phase C: 5 – 15 % for 10 minutes, 15 – 34 % for 14 minutes, 34 – 95 % for 1 minute, 95 % for 1 minute, and 95 – 5 % for 1 minute. Mobile phase A was kept at 5 % all the time.

The column eluate was on-line mixed with three-volume equivalents of Flo-Scint III liquid scintillation cocktail (PerkinElmer) at the flow rate of 1.8 mL×min^{-1}. The mixture was monitored using Ramona 2000 on-line flow-through radioactivity detector (Raytest GmbH). Retention times of detected peaks were compared with those of authentic standards to identify radio-labelled cytokinin metabolites in samples.

4.4.7 Accumulation Assays of Radio-Labelled Cytokinins in BY-2 Cell Suspension Cultures

The accumulation assays were performed according to [221], with modifications for BY-2 cell suspension cultures described in [222], [223]. The required volume of fresh BY-2 medium was inoculated as described in 4.4.1. Suspensions of transgenic cell lines were induced by estradiol. As a negative control, transgenic cell line suspensions in which an equivalent amount of dimethylsulfoxide (DMSO) was added instead of estradiol solution were used.

Two-day old BY-2 cell suspensions were filtered and resuspended in the uptake buffer, consisting of 20 mmol×L^{-1} 2-(*N*-morpholino)ethanesulfonic acid (MES), 10 mmol×L^{-1} sucrose, 0.5 mmol×L^{-1} calcium sulphate, with pH adjusted to 5.7. Resuspended cells were incubated on IS-971R orbital shaker (Jeio Tech) at 1×g (150 RPM) and 27 °C in the dark for 45 minutes. Following the first incubation, the cell suspensions were filtered and resuspended in fresh uptake buffer once more and incubated under the same conditions for 90 minutes.

Basic accumulation assays and those studying the effect of *AtPUP14* heterologous expression in BY-2 cells were initiated by adding the radio-labelled tracer into the cell suspension. The tracer concentration in the suspension was 2 nmol×L^{-1}. During fifteen minutes, 0.5 mL aliquots of the suspension were withdrawn and rapidly filtered in regular time intervals (approximately 1.5 minutes).

In kinetic accumulation assays, non-labelled competitors were added to cell suspensions before the tracer. After that, the tracer was added as well. The tracer concentration in the suspension was 2 nmol×L^{-1}. After one minute, a 1 mL aliquot of the suspension was withdrawn and rapidly filtered. Such an assay was performed over a range of competitor concentrations (0 – 2 mol×L^{-1}). As for the negative control (i.e. zero competitor concentration), the equivalent amount of DMSO was added instead of the competitor.

Dry cells, which remained after the liquid phase of the suspension aliquot was filtered, were transferred into plastic scintillation vials and lysed by a 30 minute-long extraction in 500 µL of 96% (v/v) ethanol. The tracer concentration in cells was measured via the total radioactivity determination method described in 4.4.8.

4.4.8 Total Radioactivity Determination

The samples containing a radio-labelled tracer were mixed with 4 mL of Flo-Scint III scintillation cocktail (PerkinElmer) in plastic scintillation vials. The vials were closed and shaken on KS 130 orbital shaker (IKA) at 1×g (480 RPM). Total radioactivity in samples was measured using TRI-CARB 4910TR 110V scintillation counter (PerkinElmer).

4.4.9 Mathematical Modelling of Cytokinin Membrane Transport

To describe cytokinin membrane transport kinetics, a mathematical model based on competitive inhibition kinetics (7) was employed [224]. Originally, this model was derived for a single transporter. Later, it was used to characterize membrane transport of auxins and therefore modified to account for the net effect of all transport processes on the membrane [109], [225]. The term transport system is used to refer to such a set of membrane transport processes. The kinetic model can be expressed as:

$$v = \frac{V_{max}[S]}{K_M \times \left(1 + \frac{[I]}{K_I}\right) + [S]} + NSR \quad , \tag{8}$$

where v denotes the transport rate (mol×L^{-1}×s^{-1}), ρ the cell suspension density (L^{-1}), V_{max} the maximal transport rate (mol×L^{-1}×s^{-1}), $[S]$ the substrate concentration (mol×L^{-1}), and $[I]$ the inhibitor concentration (mol×L^{-1}). K_M (mol × L^{-1}) is a function parameter which is related to the membrane transport system affinity toward the substrate. K_I (mol×L^{-1}) is a parameter related to the membrane transport system affinity toward the inhibitor. NSR (standing for the non-saturable component), is an absolute accounting for the simple diffusion rate, which also contributes to v value. It can be shown that in the absence of both the inhibitor and simple diffusion, K_M represents the substrate concentration at which the transport rate is equal to the half of its maximum value:

$$v = \frac{V_{max}}{2} \ \wedge \ [I] = 0 \ \wedge \ NSR = 0 \ \Rightarrow \ [S] = K_M \quad . \tag{9}$$

This makes K_M formally similar to the classical Michaelis constant. However, its definition in terms of rate constants differs [224].

Mathematical model (8) was firstly used to evaluate BY-2 cell membrane transport system affinity toward tZ and iP. Kinetic accumulation assays, as described in 4.4.7 were performed using radio-labelled cytokinins, whose concentration was kept constant and their non-labelled counterparts, whose concentration varied. The detected radioactivity

decreased with the increasing non-labelled cytokinin concentration, similarly to actual competitive inhibition. In such a setting, it was assumed that:

$$K_M \approx K_I \quad ; \tag{10}$$

therefore, equation (8) can be simplified to:

$$v = \frac{V_{max}[S]}{K_M + c} + NSR \quad , \tag{11}$$

where

$$c = [I] + [S] \quad . \tag{12}$$

Model (11) was used to estimate V_{max}, K_M, and NSR values for tZ and iP accumulation in BY-2 cells.

Next, model (8) was used to examine if tZ and iP compete over binding sites within the BY-2 membrane transport system. Since tZ was arbitrarily chosen to be treated as the substrate and iP as the inhibitor, the K_I value was initially predicted to be equal to K_M of iP. The actual K_I value was subsequently evaluated in a kinetic accumulation assay employing radio-labelled tZ and non-labelled iP. Numerical evaluation of the aforementioned functional parameters and estimation of their standard errors was performed using SciPy software [226].

5 Results

5.1 Transport of Radio-Labelled Cytokinins in Cell Suspension Cultures

5.1.1 Accumulation Assays of Cytokinin Free Bases and Glucosides in BY-2 (Tobacco) Cells

Accumulation assays of radio-labelled plant hormones in cell cultures are readily used to examine whether the said hormones do pass through biological membranes, as well so to further characterize their membrane translocation [222], [223], [225].

The basic accumulation protocol (as described in 4.4.7) consists in the application of a defined amount of a radio-labelled tracer to the cell suspension and subsequent measurement of total radioactivity in suspension samples taken in regular time intervals. The results of such an experiment yield information about the temporal accumulation of the radio-labelled tracer in the cells, the so-called accumulation curve.

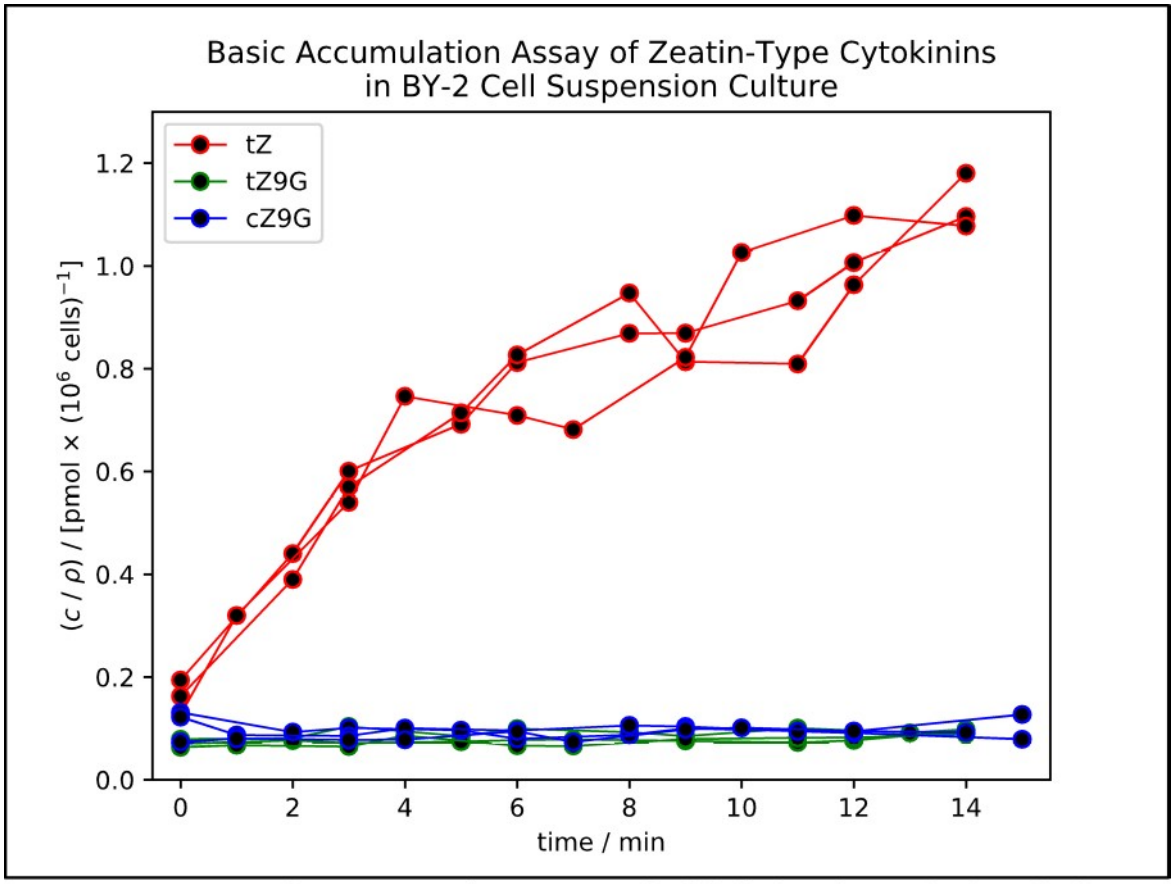

Figure 7: Basic accumulation assays of zeatin-type radio-labelled cytokinins, [3H]-tZ, [3H]-tZ9G and [3H]-cZ9G in BY-2 cell suspension cultures. The initial concentration of each cytokinin in the cell suspension culture was 2 nmol×L^{-1}. Total radioactivity detected in cells was converted to tracer concentration (c) divided by cell suspension density (ρ) and plotted against accumulation time. Each experiment was performed in three independent runs, which are presented individually.

This basic accumulation assay can be further modified. For instance, to evaluate the role of a specific membrane transporter, the expression of the corresponding genes is altered [141], [144]–[146]. To study substrate specificity of a transporter-mediated transport process, transport assays are conducted in the presence of an inhibitor [145], [146]. A similar setting can be used to confirm or disprove putative kinetic effects of a potential inhibitor, and so on.

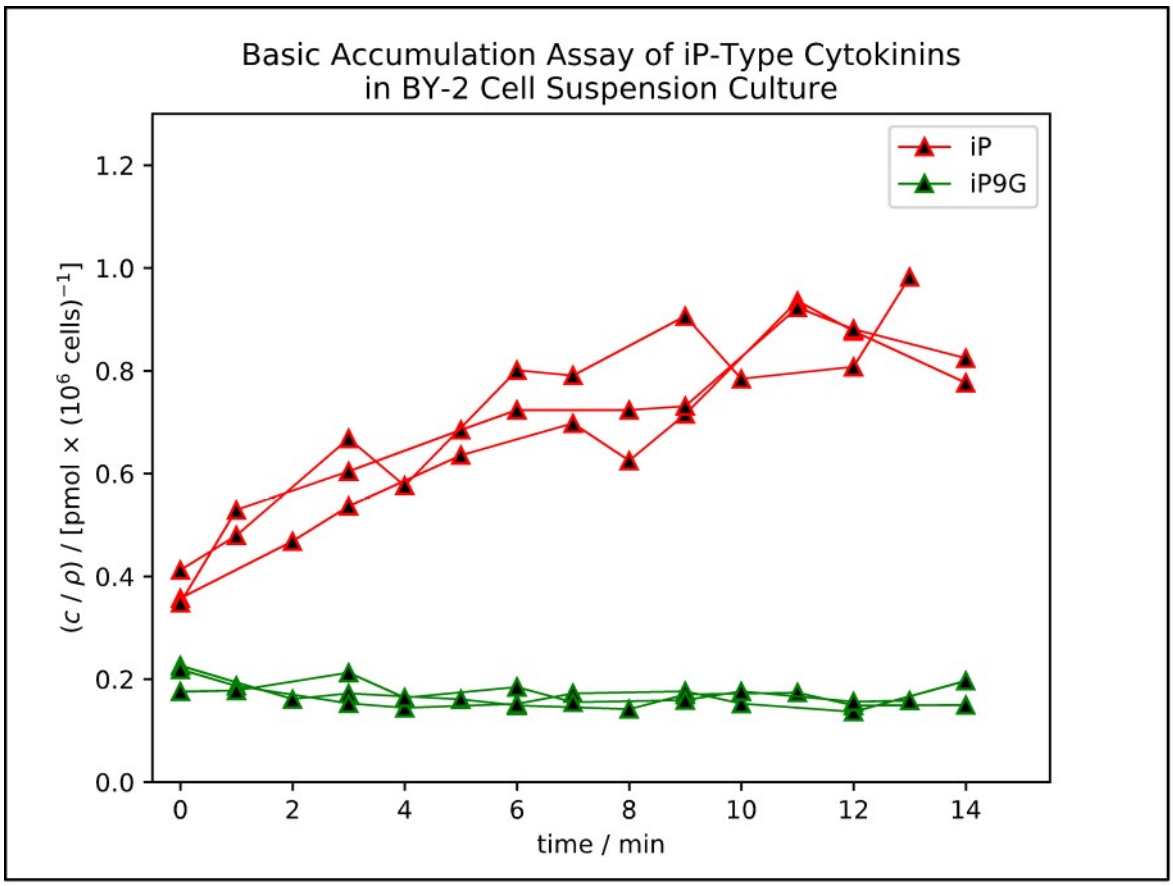

Figure 8: Basic accumulation assays of iP-type radio-labelled cytokinins, [3H]-iP and [3H]-iP9G in BY-2 cell suspension cultures. The initial concentration of each cytokinin in the cell suspension culture was 2 nmol×L^{-1}. Total radioactivity detected in cells was converted to tracer concentration (c) divided by cell suspension density (ρ) and plotted against accumulation time. Each experiment was performed in three independent runs, which are presented individually.

Since the membrane transport of glycosylated cytokinins hasn't been studied profoundly yet, we decided to begin our experiments with basic accumulation essays of tritium-labelled cytokinins in form of N^9-glucosides, namely [3H]-tZ9G, [3H]-iP9G and [3H]-cZ9G. To be able to put the eventual results into a wider context, the accumulation assays were performed with [3H]-tZ and [3H]-iP as well.

The accumulation curves of zeatin-type radio-labelled cytokinins are depicted in Figure 7 and those of iP-type cytokinins in Figure 8. Both free bases, tZ and iP, were readily accumulated by the cell suspension, as the detected amount of radio-labelled

substance increased in fifteen minutes. Therefore, tZ and iP are transported through the biological membrane into cells.

On the other hand, the detected amount of radio-labelled glucosides remained almost constant and very low. Comparisons among accumulation curves of free bases and glucosides show that the average detected amount of radio-labelled glucosides was always less than or equal to the initial detected amount of radio-labelled bases. It is therefore likely that in the case of cytokinin glucosides, the detected radioactivity corresponds to surface contamination of the cells and that no actual membrane transport process occurred in fifteen minutes.

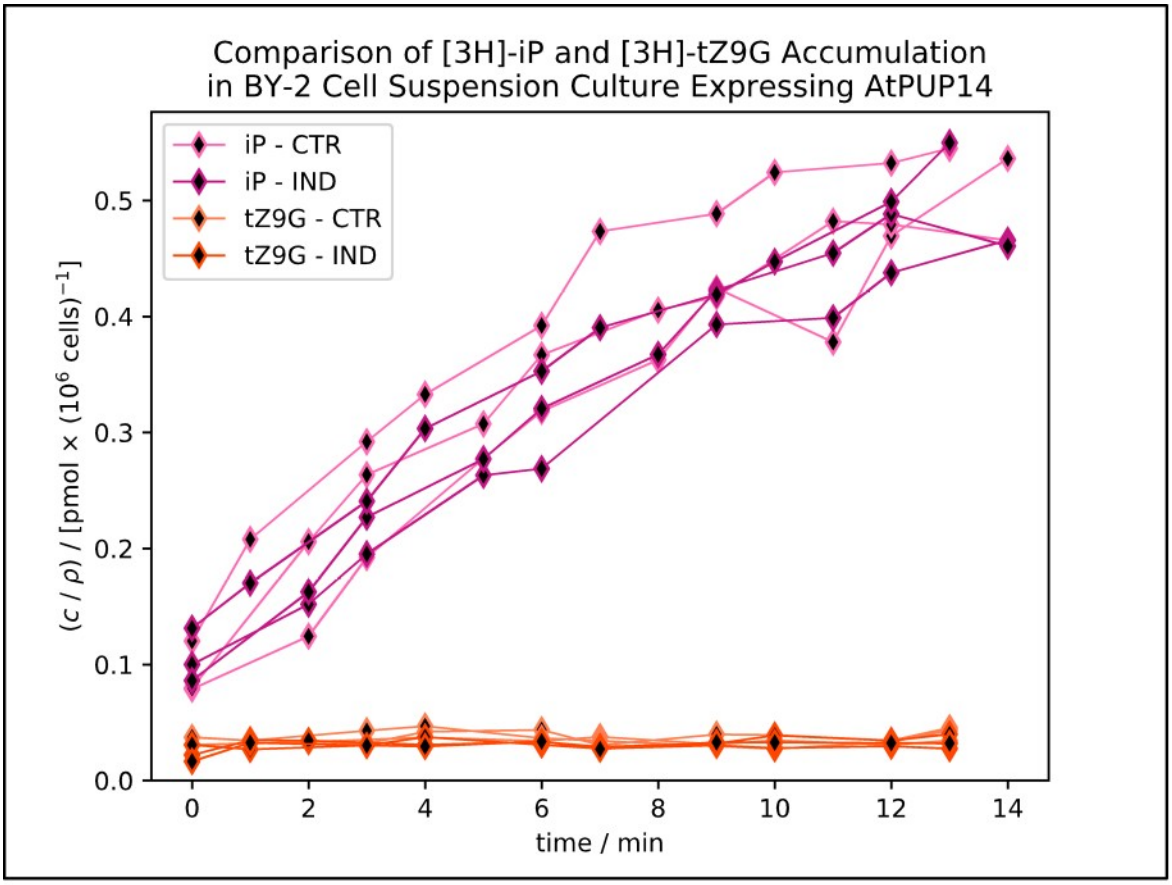

Figure 9: Accumulation assays of [3H]-iP and [3H]-tZ9G in transgenic BY-2 cell suspension culture expressing AtPUP14 gene. The initial concentration of each cytokinin in the cell suspension culture was 2 nmol×mol^{-1}. Total radioactivity detected in cells was converted to tracer concentration (c) divided by cell suspension density (ρ) and plotted against accumulation time. Each experiment was performed in three independent runs, which are presented individually. Experimental runs labelled as CTR were conducted in cells that did not express AtPUP14, due to a lack of the inductor, whereas experimental runs labelled as IND were conducted in cells in which the expression of AtPUP14 had been induced beforetheir growth.

5.1.2 Accumulation Assays of iP and Cytokinin Glucosides in BY-2 Cells Expressing *AtPUP14*

Experiments described in 5.1.1 were conducted in wild-type cell suspension cultures. Therefore, all accumulation of radio-labelled tracers occurred due to passive

diffusion or transport mediated by membrane-bound carriers native to the given cell type. Since wild-type cells did not accumulate any cytokinin glucosides, we repeated the accumulation assays in transgenic BY-2 cell suspension cultures expressing *AtPUP14*, whose product was identified as tZ-specific transporter.

Alongside either [3H]-tZ9G, [3H]-iP9G and [3H]-cZ9G, free base [3H]-iP was accumulated in each cell suspension culture as well, serving as a positive control. The expression of *AtPUP14* was inducible, allowing to perform each accumulation assay in both induced and non-induced transgenic BY-2 cell suspension cultures, the latter serving as a negative control.

In all three cases, the detected amount of [3H]-iP increases in cells over time, whereas the amount of glucoside tracers remains more or less constant and very low, similarly to results obtained for accumulation assays in wild-type cell suspension cultures. Once again, it is probable that the radioactivity detected after accumulation assays of radio-labelled cytokinin glucosides originates in superficial contamination of cells, rather than in actual membrane transport of the glucosides. Because of this, only results for [3H]-iP and [3H]-tZ9G accumulation in *AtPUP14*-expressing BY-2 cells are shown. They are depicted in Figure 9.

No significant difference between accumulations of either [3H]-iP or radio-labelled cytokinin glucosides in induced *AtPUP14*-expressing cell lines and accumulations in control lines was observed. Therefore, following experiments in cell suspension cultures were performed in wild-type cell lines only.

5.1.3 Kinetic Accumulation Assays of tZ and iP in BY-2 Cells and Evaluation of Their Respective Kinetic Parameters Using Mathematical Modelling

These accumulation curves represent net results of all transport processes of the given substances at biological membranes, but they do not yield any information about which types of membrane transport (as summarized in 2.4) occur in the cell suspension cultures and how much they participate to the total transport rate. To see if membrane transporters are involved in radio-labelled cytokinin translocation, another type of accumulation assay was performed. This time, radio-labelled cytokinins were accumulated by BY-2 cell suspension cultures in the presence of their non-labelled counterparts, which formally act as competitors. This type of accumulation was stopped after one minute to see the effects of different competitor concentrations on the initial total transport rate. The

measured rates were divided by cell density, ρ (L^{-1}), so that they could be treated as intensive, rather than extensive properties of BY-2 cells. It follows that parameters V_{max}

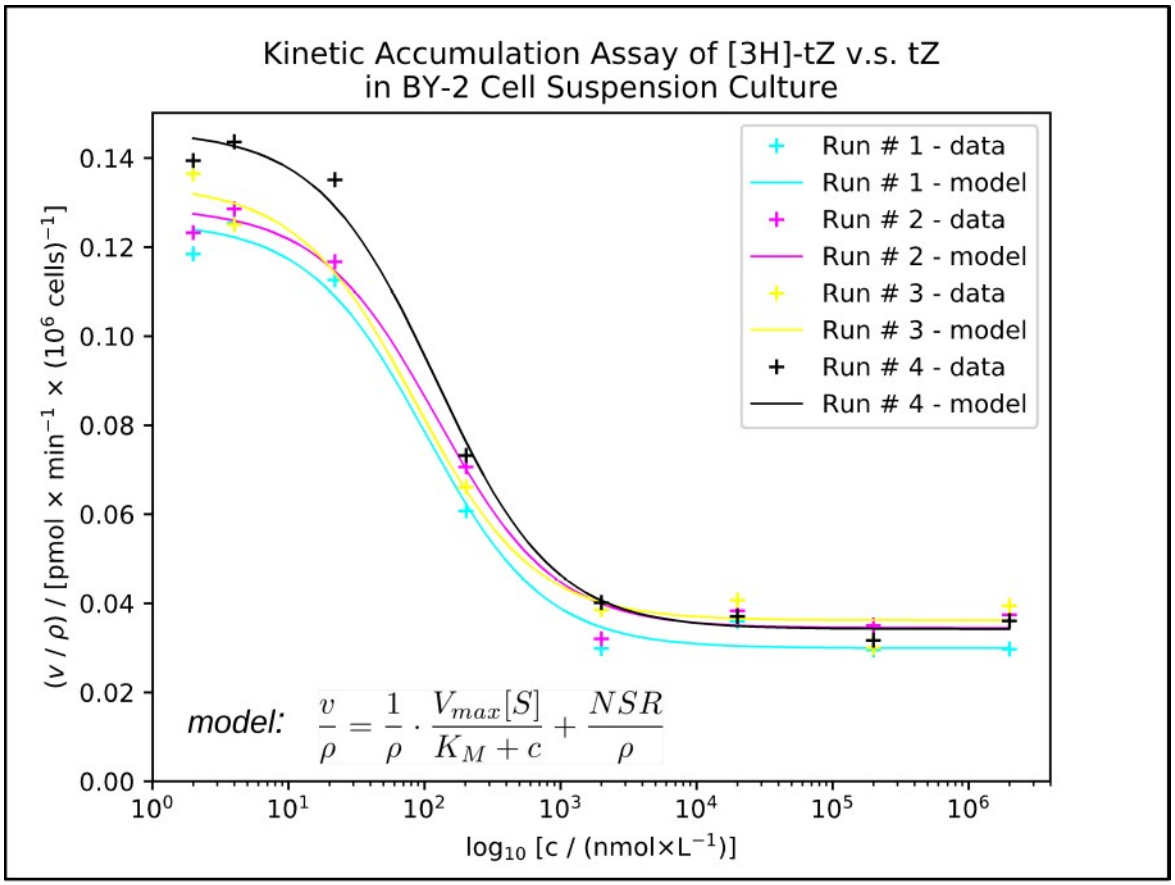

Figure 10: Kinetic accumulation assay of tZ. Radio-labelled tracer [3H]-tZ was accumulated in BY-2 cell suspension cultures in presence of its non-labelled counterpart for one minute. The radio-labelled tracer concentration, [S], remained constant, while the non-labelled tZ concentration varied. Therefore, the sum concentration of both tZ forms, c, varied as well. Total radioactivity detected in cells was converted to transport rate (v) divided by cell suspension density (ρ) and plotted against the sum concentration. The raw experimental data were fitted by the mathematical model given in the figure. The assay was performed in four independent runs, which are presented individually. Evaluated kinetic parameters are given in Table 5.

and NSR were evaluated in forms of V_{max} / ρ and NSR / ρ, respectively.

Experimental values of v / ρ were plotted against the decimal logarithm of $[S]$ and $[I]$. Such a plot was expected to yield a decreasing sigmoid curve. Thus, fitting experimental data with the appropriate model enabled both to decide whether a cytokinin-specific transport system is present, and to evaluate the model parameters.

At first, such competition assays were conducted for [3H]-tZ versus non-labelled tZ and [3H]-iP versus non-labelled iP to evaluate respective affinities of the BY-2 membrane transport system toward the two free bases, expressed in terms of K_M. To interpolate V_{max} / ρ, K_M, and NSR / ρ parameters, mathematical model (11) was used.

The results of these two assays are depicted in Figure 10 for tZ and in Figure 11 for iP. In both cases, the expected sigmoid curve could be fitted into experimental data, indicating that both tZ and iP are at least partly transported via membrane-bound carriers in BY-2 cell suspension culture.

Kinetic parameters for the accumulation of radio-labelled cytokinins in BY-cell suspension culture are given in Table 5 for [3H]-tZ and in Table 6 for [3H]-iP. The mean values of K_M were evaluated as 108.31 ± 24.84 (tZ) and 64.84 ± 29.07 nmol×L^{-1} (iP). Such a difference may suggest that the membrane transport system in BY-2 cell suspension culture has a higher affinity toward iP than toward tZ. However, given the relatively large error values, such a conclusion should not be firmly drawn unless supported by other experimental results.

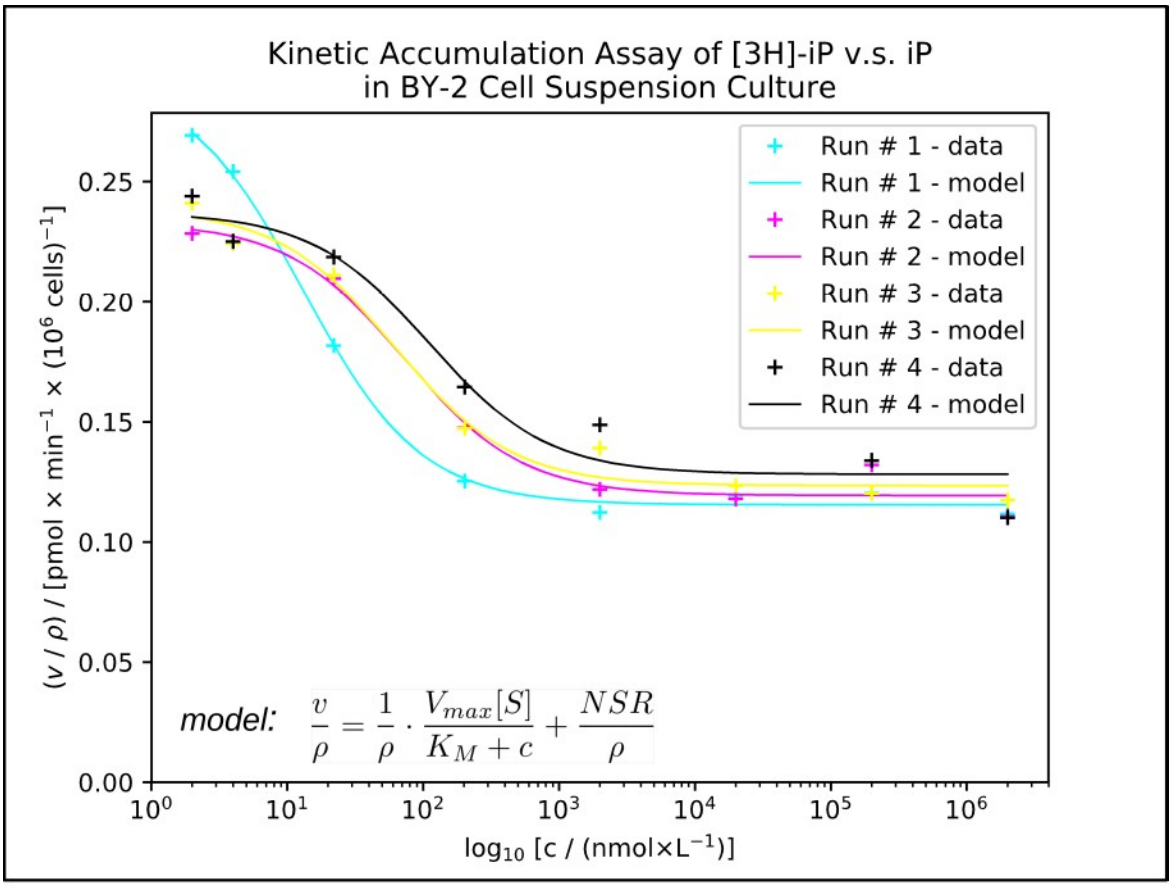

Figure 11: Kinetic accumulation assay of iP. Radio-labelled tracer [3H]-iP was accumulated in BY-2 cell suspension cultures in presence of its non-labelled counterpart for one minute. The radio-labelled tracer concentration, [S], remained constant, while the non-labelled iP concentration varied. Therefore, the sum concentration of both iP forms, c, varied as well. Total radioactivity detected in cells was converted to transport rate (v) divided by cell suspension density (ρ) and plotted against the sum concentration. The raw experimental data were fitted by the mathematical model given in the figure. The assay was performed in four independent runs, which are presented individually. Evaluated kinetic parameters are given in Table 6.

Table 5: Kinetic parameters of tZ accumulation in BY-2 cell suspension culture. The parameters were evaluated by fitting mathematical model (11) to the experimental plot of [3H]-tZ transport rate values against the total concentration of tZ in the cell suspension culture. The experiment was performed in four independent runs. Results of each run are presented individually. In the last column, mean parameter values over all four runs are given.

Kinetic Parameters of tZ Accumulation in BY-2 Cells					
Parameter	**Run #1**	**Run #2**	**Run #3**	**Run #4**	**Mean**
V_{max} / ρ [pmol×min^{-1} × (10^6 cells)$^{-1}$]	4.95 ± 1.12	5.79 ± 1.32	4.20 ± 1.25	6.81 ± 1.16	**5.44 ± 1.21**
K_M [nmol×L^{-1}]	103.29 ± 24.09	122.62 ± 28.47	85.70 ± 25.70	121.54 ± 21.10	**108.31 ± 24.84**
NSR / ρ [fmol×min^{-1} × (10^6 cells)$^{-1}$]	29.90 ± 2.46	34.44 ± 2.49	36.15 ± 2.80	34.18 ± 2.20	**33.7 ± 2.49**

Table 6: Kinetic parameters of iP accumulation in BY-2 cell suspension culture. The parameters were evaluated by fitting mathematical model (11) to the experimental plot of [3H]-iP transport rate values against the total concentration of iP in the cell suspension culture. The experiment was performed in four independent runs. Results of each run are presented individually. In the last column, mean parameter values over all four runs are given.

Kinetic Parameters of iP Accumulation in BY-2 Cells					
Parameter	**Run #1**	**Run #2**	**Run #3**	**Run #4**	**Mean**
V_{max} / ρ [pmol×min^{-1} × (10^6 cells)$^{-1}$]	1.17 ± 0.13	4.17 ± 1.43	3.56 ± 1.17	6.05 ± 3.57	**3.74 ± 1.58**
K_M [nmol×L^{-1}]	13.07 ± 1.75	73.35 ± 27.01	61.96 ± 21.33	110.97 ± 66.17	**64.84 ± 29.07**
NSR / ρ [fmol×min^{-1} × (10^6 cells)$^{-1}$]	115.43 ± 1.86	119.29 ± 4.14	123.43 ± 3.97	128.15 ± 8.26	**121.57 ± 4.56**

The mean values of NSR / ρ can be used to evaluate how much is the transport rate affected by a component independent on membrane carriers, i.e. simple diffusion. The obtained mean values of NSR / ρ , 33.7 ± 2.49 (tZ) and 121.57 ± 4.56 fmol×min^{-1} per a million cells (iP), show that iP diffuses across membranes with a greater rate than tZ. Nevertheless, the mean NSR / ρ values make up only 0.6% (tZ) and 3.2% (iP) of obtained mean V_{max} / ρ values (5.44 ± 1.21 for tZ and 3.74 ± 1.58 pmol×min^{-1} per a million cells for iP), suggesting that tZ and iP uptakes are mostly mediated by membrane carriers.

Having seen that both tZ and iP enter BY-2 cells via carrier-mediated transport, we continued by exploring the possible competition between the two bases. Existence of such competition would mean that tZ and iP are transported at least partially via the same

system. We performed another kinetic accumulation assay, this time using [3H]-tZ as the tracer and non-labelled iP as the competitor. Experimental data were fitted with the mathematical model (8), using the previously evaluated mean value of K_M for tZ

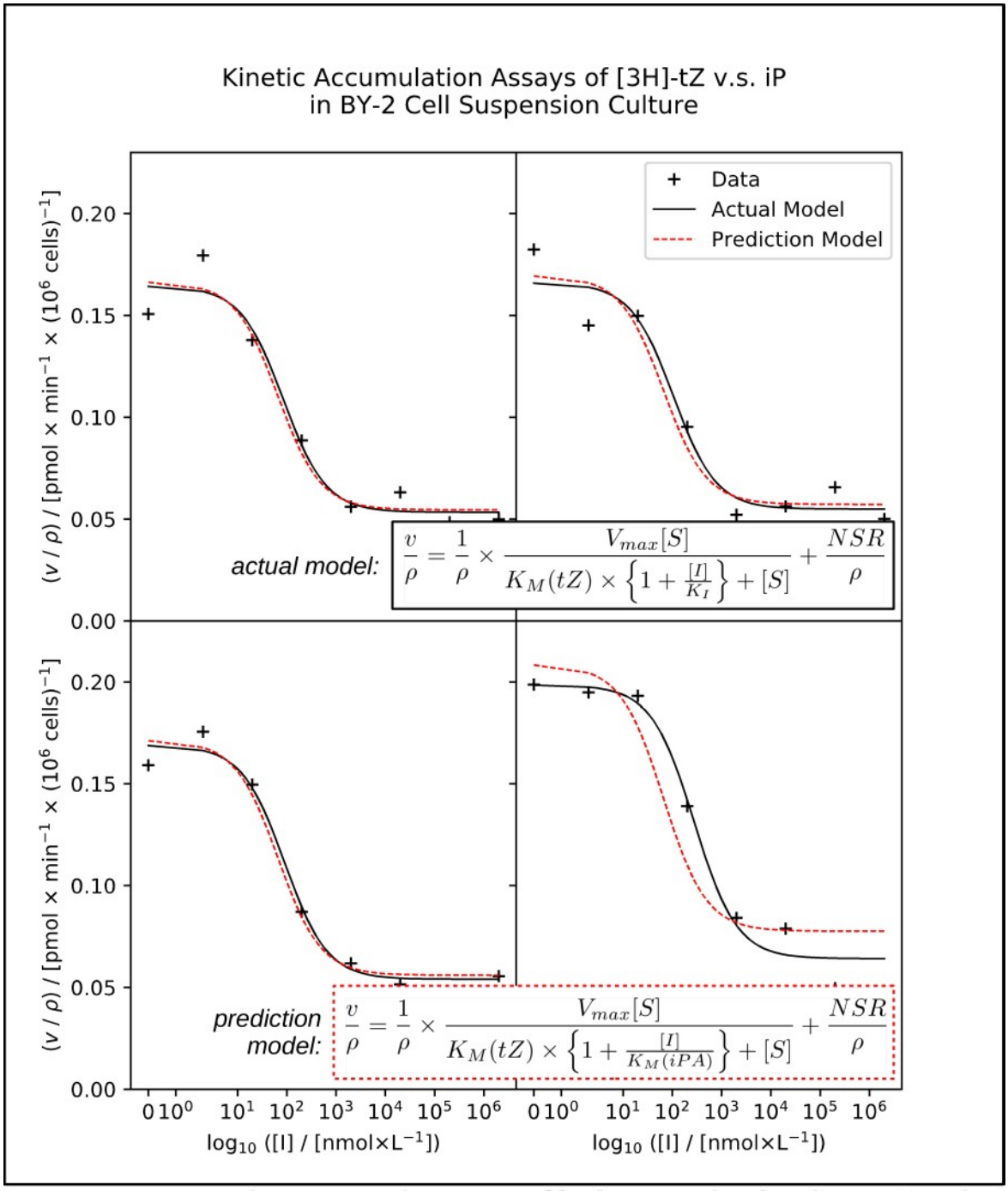

Figure 12: Competition kinetic accumulation assay of [3H]-tZ, treated as the substrate (S), and non-labelled iP, treated as the competitive inhibitor (I), in BY-2 cell suspension culture. Each accumulation lasted for one minute. The tracer's concentration was 2 nmol×L^{-1}, the inhibitor's concentration varied. Total radioactivity detected in cells was converted to transport rate (v) divided by cell suspension density (ρ) and plotted against the inhibitor concentration. The raw experimental data were fitted by two mathematical models given in the top and the bottom part of the figure, respectively. Each experiment was performed in four independent runs, which are presented as individual plots. Evaluated kinetic parameters are given in Table 7

accumulation in BY-2 cells. To confirm the model validity, experimental data were initially fitted with a model where the K_I value was guessed to be equal to K_M of iP accumulation in BY-2 cells (see Figure 12). From this fit, values of V_{max} / ρ and NSR / ρ were predicted. Then, the actual values of K_I, V_{max} / ρ, and NSR / ρ were determined by fitting experimental data with the unmodified model (8). In Figure 12, a comparison of both prediction and actual model is shown for four independent experimental runs. Kinetic parameters evaluated for both models are given in Table 7.

Table 7: Kinetic parameters of tZ accumulation in BY-2 cell suspension culture in the presence of iP acting as a competitive inhibitor. The parameters were evaluated by fitting model (8) to the experimental plot of [3H]-tZ transport rate values against the concentration of non-labelled iP in the cell culture. Predicted parameters (top) were obtained by considering the mean value of $K_M(tZ)$ (see Table 5) as K_M and the mean value of $K_M(iPA)$ (see Table 6) as K_I. Actual parameters (bottom) were obtained when no value was a priori assigned to K_I.

Comparison of Prediction and Actual Model of [3H]-tZ Accumulation in BY-2 Cells in the Presence of iP					
Parameter	***Prediction***				
	Run #1	**Run #2**	**Run #3**	**Run #4**	**Mean**
V_{max} / ρ [pmol×min^{-1} × (10^6 cells)$^{-1}$]	6.16 ± 0.49	6.18 ± 0.58	6.35 ± 0.36	7.21 ± 0.99	*6.48 ± 0.61*
NSR / ρ [fmol×min^{-1} × (10^6 cells)$^{-1}$]	54.56 ± 5.07	57.21 ± 5.95	55.98 ± 3.92	77.61 ± 10.87	*61.34 ± 6.45*
Parameter	***Evaluation***				
	Run #1	**Run #2**	**Run #3**	**Run #4**	**Mean**
V_{max} / ρ [pmol×min^{-1} × (10^6 cells)$^{-1}$]	10.11 ± 0.87	10.12 ± 0.97	10.47 ± 0.58	12.24 ± 0.83	**10.73 ± 0.81**
K_I [nmol×L^{-1}]	83.86 ± 40.80	103.75 ± 55.50	88.97 ± 26.81	274.23 ± 96.90	**137.70 ± 55.0**
NSR / ρ [fmol×min^{-1} × (10^6 cells)$^{-1}$]	53.37 ± 5.95	54.89 ± 6.77	53.99 ± 4.38	64.16 ± 6.85	**56.60 ± 5.99**

Actual evaluated values of both V_{max} / ρ and NSR / ρ are fairly similar to the respective predictions, suggesting that a transport model considering tZ and iP as competitive inhibitors on biological membranes is valid. Due to large errors of the evaluated K_I parameter values, the competition effect of iP on tZ uptake by BY-2 cells cannot be further discussed.

5.2 Metabolism of Radio-Labelled Cytokinins in *Arabidopsis* Plants

5.2.1 Cytokinin Uptake Mediated by *Arabidopsis* Plants

The previous experiments were done in tobacco BY-2 cell suspension cultures. Such systems, while offering advantages of easy manipulation and upscaling, allow us to observe only those physiological processes that are independent of positional information, tissue or organ specificity and so on. Furthermore, when exploring functionalities of *Arabidopsis* genes (such as *AtPUP14* or *AtABCG14*), transgenic tobacco lines act as heterologous expression systems, potentially employing different post-translational modifications.

Therefore, we performed another set of experiments, this time on 14-day old *Arabidopsis* plants. The plants were treated with 20nM solutions of radio-labelled cytokinins [3H]-tZ, [3H]-iP, [3H]-tZ9G, [3H]-iP9G and [3H]-cZ9G. The treatment lasted for 100 minutes. The experiment was performed using wild-type (WT) plants, *atabcg14* and *atpup14* mutant plants, and *AtUGT76C2*-overexpressing plants.

At first, we examined the total uptake of radio-labelled tracer by *Arabidopsis* plants. Each tracer's uptake was evaluated as the ratio between the tracer concentrations detected in the corresponding media before and after the plant treatment. In media used to treat WT plants, [3H]-tZ concentration dropped to about 60% and [3H]-iP concentration to about 80% of the corresponding initial concentrations. In media used to treat *atabcg14* and *atpup14* plants, the final concentrations of both radio-labelled bases were about 80% and 90% of the corresponding initial concentrations, respectively.

No significant difference between the initial and final concentration of radio-labelled cytokinin glucosides was observed in either case, suggesting that they were taken either in a very small amount or not at all. An exception to this observation was the medium used to treat *atabc14* plants with [3H]-iP9G, where the final concentration was about 120% of the initial one. Such an increase most likely occurred due to contamination of the medium sample during total radioactivity analysis. The differences between initial and final concentrations of radio-labelled cytokinin tracers in media are depicted in Figure 13.

In media used to treat *AtUGT76C2*-overexpressing plants, the final concentration of neither tracer changed in respect to the initial one, suggesting that these plants didn't take

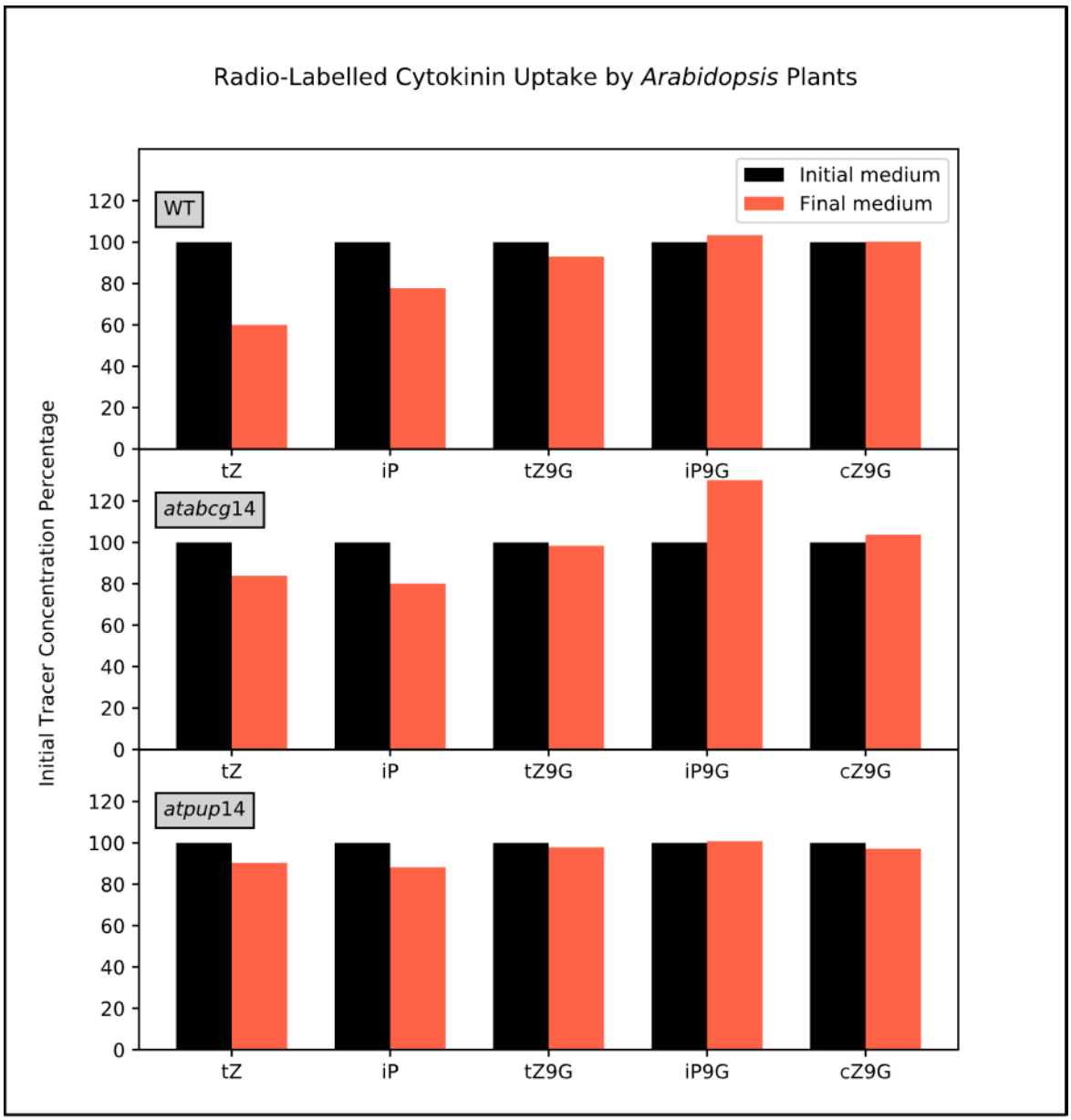

Figure 13: Radio-labelled cytokinin uptake by Arabidopsis plants. Each pair of bars represent a difference between tracer concentration in MS/2 media before (black) and after 100-minute plant treatment (orange). The tracer concentrations are expressed as the initial concentration percentage. WT: wild type.

even cytokinin bases (data not shown). Since these results probably stem from unsatisfactory plant conditions or experiment set-up, they were further considered invalid.

5.2.2 Identification of Cytokinin Metabolites in *Arabidopsis* Plants

Treated plants and media samples (taken both before and after the treatment) were further analysed by high-performance liquid chromatography (HPLC) to identify metabolites of the radio-labelled tracers and to express their relative amounts. Before analysis, cytokinin fractions were isolated from plant samples, according to a method developed by P. Dobrev and M. Kamínek [169].

Metabolic conversions of cytokinins may result in concentration change of those forms that are carried over biological membranes. Since both equilibrium constant and transport rate depend on substrate concentrations on either side of the membrane, it is clear that cytokinin metabolism affects their transport. Furthermore, changes in metabolism tied to mutation or over-expression of certain transported genes may help to further discuss the said transporter's role *in planta*. Results of metabolic assays are presented in form of tables. The area under the curve (AUC) of each peak was divided by the total AUC, yielding total AUC percentages of each detected metabolite.

Table 8: Metabolism of [3H]-tZ (trans-zeatin) and [3H]-iP (isopentenyladenine) in Arabidopsis plants. Cytokinin fraction isolated from plant material was analysed via HPLC, using Kinetex C18 column, 150 × 4.6 mm, 5 µm (Phenomenex) and Ramona 2000 on-line flow-through radioactivity detector (Raytest GmbH). For each metabolite, percentages of the total area under the curve (AUC) are given for three types of sample and three types of plants. WT: wild type; Ade: adenine, Ado: adenosine, tZRMP: trans-zeatin riboside monophosphate, tZ7G: trans-zeatin N^7-glucoside, tZ9G: trans-zeatin N^9-glucoside, tZR: trans-zeatin riboside, iP7G: isopentenyladenine N^7-glucoside, iP9G: isopentenyladenine N^9-glucoside, iPRMP: isopentenyladenine riboside monophosphate ; n .d.: not detected.

[3H]-tZ	**Total AUC Percentage [%]**								
Metabolite	**Plant Tissues**			**Media Before Treatment**			**Media After Treatment**		
	WT	*abcg14*	*pup14*	WT	*abcg14*	*pup14*	WT	*abcg14*	*pup14*
Ade	33.51	35.81	57.34	8.20	7.33	12.66	17.98	29.91	34.12
Ado	13.74	13.84	15.48	1.49	1.59	2.13	2.38	2.60	4.20
tZRMP	9.25	5.99	2.93	1.13	0.87	*n. d.*	1.66	0.70	*n. d.*
tZ7G	3.54	2.10	1.68	0.87	0.75	*n. d.*	0.98	0.82	*n. d.*
tZ9G	1.53	0.98	1.22	0.69	1.21	*n. d.*	0.67	1.40	*n. d.*
tZ	12.09	9.32	2.41	67.91	61.19	58.55	57.15	38.19	35.52
tZR	7.22	2.76	4.54	*n. d.*	3.14	*n. d.*	*n. d.*	*n. d.*	*n. d.*
[3H]-iP	**Total AUC Percentage [%]**								
Metabolite	**Plant Tissues**			**Media Before Treatment**			**Media After Treatment**		
	WT	*abcg14*	*pup14*	WT	*abcg14*	*pup14*	WT	*abcg14*	*pup14*
Ade	43.99	46.00	56.04	18.24	18.64	19.73	35.84	45.26	42.96
Ado	15.15	13.46	13.2	3.14	2.97	3.59	4.57	4.73	4.86
tZ	0.62	0.47	0.69	0.87	0.73	2.65	1.15	0.46	2.75
iP7G	5.53	3.70	2.57	2.30	2.50	2.06	2.30	1.43	1.97
iPRMP	1.55	0.52	0.49	1.06	0.53	0.35	0.94	0.39	0.68
iP9G	0.77	0.52	0.25	0.60	0.19	*n. d.*	0.77	0.23	0.47
iP	4.00	1.67	0.84	50.34	47.73	50.34	26.99	24.33	21.47

Metabolic data for [3H]-tZ and [3H]-iP are given in Table 8. In [3H]-tZ metabolic profiles, adenine, adenosine, *trans*-zeatin riboside monophosphate (tZRMP), *trans*-zeatin N^7-glucoside (tZ7G), tZ9G, tZ and *trans*-zeatin riboside (tZR) were detected. In all plant tissue samples, adenine was the most abundant metabolite in all plant tissue samples, making for 33.5 % (WT), 35.8 % (*atabcg14*), and 57.3 % (*atpup14*) of total AUC. Adenine was also present in media before the treatment, probably due to tracer decay.

During the treatment of all plant types, the relative amount of adenine in media increased. Adenosine was found mainly in plant tissue samples, making for 13.7 % (WT), 13.8 % (*atabcg14*), and 15.5 % (*atpup14*) of the total AUC; tZMRP made for 12.1 % and 9.3 % of the total AUC in WT and *atabcg14* plant tissue samples, respectively, but only 2.9 % of the total AUC in *atpup14* plant tissue samples; tZ7G made for 3.5 % of the total AUC in WT plant tissue samples. In other samples, both tZ7G and tZ9G were found in trace amounts only. Being the initial radio-labelled tracer, tZ was also the most abundant substance detected in all media samples. In all cases, its relative amount in media decreased in the course of the treatment. In plants, tZ made for 12.1 % and 9.3 % of the total AUC in WT and *abcg14* plant tissue samples, respectively, but only 2.8 % of the total AUC in *atpup14* plant tissue samples. Lastly, tZR was found mostly in plant tissue samples, making for 7.2 % (WT), 2.8 % (*atabcg14*), and 4.5 % (*atpup14*) of the total AUC.

In [3H]-iP metabolic profiles (see Table 8), adenine, adenosine, tZ, isopentenyladenine N^7-glucoside (iP7G), isopentenyladenine riboside monophosphate (iPRMP), iP9G and iP were found. Similarly to the results concerning [3H]-tZ metabolism, adenine was the most abundant metabolite in all plant tissue samples, making for 44.0 % (WT), 46.0 % (*atabcg14*), and 56.0 % (*atpup14*) of the total AUC. It was present in both initial and final media and its relative amount increased in the course of all treatments. It was also the most abundant metabolite in all final media samples. Adenosine was found mostly in plant tissue samples, making for 15.2 % (WT), 13.5 % (*atabcg14*), and 13.2 % (*atpup14*) of the total AUC. In media samples, adenosine relative amount remained below 5 % of the total AUC and there was no significant difference between AUC percentage during the treatment in either case. As for tZ, iPRMP, and iP9G, they were found only in trace amounts in all sample types. In plant tissue samples, iP7G made for 5.5 % (WT), 3.7 % (*atabcg14*), and 2.6 % (*atpup14*) of the total AUC. In all media samples, its relative amount was below 3 % of the total AUC and didn’t significantly change in the course of either plant type treatment. The original tracer, iP, was found mostly in both initial and final media. In course of the treatments, the relative amount of iP in media dropped from

50.3 % to 27.0 % (WT), from 47.7 % to 24.3 % (*atabcg14*), and from 50.3 % to 21.5 % (*atpup14*) of the total AUC. In WT plant tissue samples, iP made for 4.0 % of the total AUC, while in *atabcg14* and *atpup14* samples, it was only 1.7 % and 0.8 %, respectively.

Table 9: Metabolism of [3H]-tZ9G (trans-zeatin N^9-glucoside), [3H]-iP9G (isopentenyladenine N^9-glucoside), and [3H]-cZ9G (cis-zeatin N^9-glucoside) in Arabidopsis plants. Cytokinin fraction isolated from plant material was analysed via HPLC, using Kinetex C18 column, 150 × 4.6 mm, 5 µm (Phenomenex) and Ramona 2000 on-line flow-through radioactivity detector (Raytest GmbH). For each metabolite, percentages of the total area under the curve (AUC) are given for three types of sample and three types of plants. WT: wild type; Ade: adenine, Ado: adenosine, tZRMP: trans-zeatin riboside monophosphate, tZ7G: trans-zeatin N^7-glucoside, tZ: trans-zeatin, tZR: trans-zeatin riboside; n. d.: not detected.

[3H]-tZ9G	**Total AUC Percentage [%]**								
Metabolite	**Plant Tissues**			**Media Before Treatment**			**Media After Treatment**		
	WT	*abcg14*	*pup14*	WT	*abcg14*	*pup14*	WT	*abcg14*	*pup14*
Ade	2.65	1.48	3.21	1.44	2.67	2.36	1.38	4.59	0.58
Ado	1.04	1.07	0.68	0.91	0.59	1.21	2.26	0.90	0.63
tZRMP	0.33	0.72	*n. d.*	0.41	0.56	*n. d.*	0.60	0.76	*n. d.*
tZ7G	0.50	*n. d.*	2.02	0.38	*n. d.*	*n. d.*	1.47	*n. d.*	*n. d.*
tZ9G	85.53	94.06	88.11	78.54	93.30	80.89	70.55	88.15	92.63
tZ	1.65	*n. d.*	*n. d.*	2.06	*n. d.*	*n. d.*	1.55	*n. d.*	*n. d.*
tZR	1.16	*n. d.*	*n. d.*	1.11	*n. d.*	*n. d.*	1.62	*n. d.*	*n. d.*
[3H]-iP9G	**Total AUC Percentage [%]**								
Metabolite	**Plant Tissues**			**Media Before Treatment**			**Media After Treatment**		
	WT	*abcg14*	*pup14*	WT	*abcg14*	*pup14*	WT	*abcg14*	*pup14*
Ade	*n. d.*	0.67	1.40	*n. d.*	0.46	2.54	*n. d.*	0.47	0.54
Ado	*n. d.*	0.48	0.14	*n. d.*	0.29	0.47	*n. d.*	0.18	0.08
iP9G	88.45	91.31	87.15	83.48	89.76	86.13	76.21	93.97	91.49
[3H]-cZ9G	**Total AUC Percentage [%]**								
Metabolite	**Plant Tissues**			**Media Before Treatment**			**Media After Treatment**		
	WT	*abcg14*	*pup14*	WT	*abcg14*	*pup14*	WT	*abcg14*	*pup14*
Ade	*n. d.*	0.61	1.84	*n. d.*	*n. d.*	2.18	*n. d.*	*n. d.*	*n. d.*
cZ9G	88.28	89.71	84.95	82.69	89.68	75.32	77.70	88.96	88.69

Metabolic data for [3H]-tZ9G, [3H]-iP9G, and [3H]-cZ9G are given in Table 9. In [3H]-tZ9G metabolic profiles, tZ metabolites listed above were detected. However, other metabolites than tZ9G were present in trace relative amounts only. In plant tissue samples, tZ9G made for 85.5 % (WT), 94.1 % (*atabcg14*), and 88.1 % (*atpup14*) of the total AUC. In media samples, there was no significant difference between relative amounts of tZ9G

before and after the treatment in either case. As for the two other glucosides, [3H]-iP9G and [3H]-cZ9G, conclusions similar to those for [3H]-tZ9G can be made.

In [3H]-iP9G metabolic profiles (see Table 9), only trace amounts of adenine and adenosine were found alongside iP9G itself. In plant tissue samples, iP9G made for 88.3 % (WT), 91.3 % (*atabcg14*) and 87.2 % (*atpup14*) of the total AUC.

In [3H]-cZ9G metabolic profiles (see Table 9), only cZ9G and trace amounts of adenine were found. In plant tissue samples, cZ9G made for 88.3 % (WT), 89.7 % (*atabcg14*) and 85.0 % (*atpup14*) of the total AUC.

In media samples, relative amounts of neither glucoside significantly changed in course of neither plant treatment. All things considered, no significant metabolic processes were observed when treating *Arabidopsis* plants with radio-labelled cytokinin glucosides. Moreover, given their hardly detectable uptakes by the plants, the detected glucosides might have occurred in the samples due to superficial contamination, rather than actual transport processes.

5.3 Cytokinin Transporter Mutation Effects on *De Novo* Tissue Formation in *Arabidopsis* Hypocotyls

5.3.1 *De Novo* Tissue Formation in *Arabidopsis* Hypocotyls Muted in Various Cytokinin Transport-Related Genes After Kinetin and Auxin (3 : 1) Treatment

The last set of experiments was based on reports published by Kubo and Kakimoto (2000) [220] and Pernisová et al. (2009) [219]. In these works, the authors studied organogenesis mediated in *Arabidopsis* hypocotyl explants by auxin and cytokinin cross-talk. One of the experimental procedures they used consists in treating hypocotyls excised from 14-day old *Arabidopsis* seedlings with exogenous kinetin and auxin, causing either formation of either undifferentiated calli, shoot-like or root-like tissues. As shown in [219], [220], the results of such assays depend on the ratio between auxins and cytokinins and can be altered by some genetic modification, such as mutations in genes regulating cytokinin signalization pathway.

We adopted these assays to explore potential effects of putative cytokinin transporter genes on said organogenetic processes and to see whether such an assay could be generally used as a tool in further cytokinin transport research.

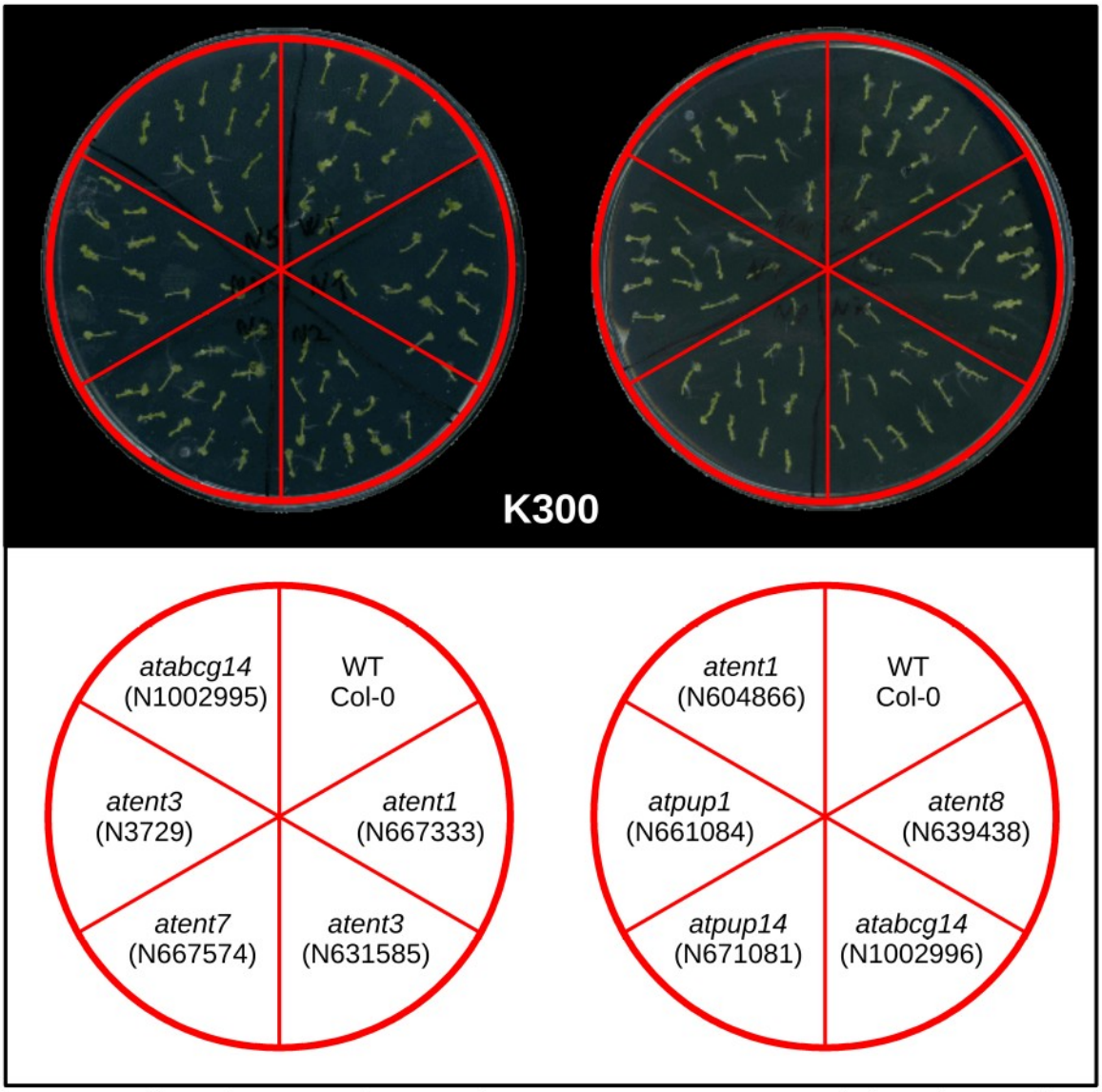

Figure 14: Effects of exogenous kinetin and auxin on organogenesis in Arabidopsis hypocotyl explants. The explants were cultivated on media K300, consisting of 300 µg×L^{-1} kinetin and 100 µg×L^{-1} auxin (1-naphthaleneacetic acid). Each Petri dish with the K300 medium was divided into six parts. Each part contained either wild-type (WT) hypocotyl explants (ecotype Columbia, or Col-0) or one of the single-gene mutants, according to the schemes in the lower part of the figure. For mutant lines, NASC accession numbers are given in parentheses.

To begin with, we performed a scanning assay, treating hypocotyls from several single-gene mutant *Arabidopsis* plants with kinetin and auxin in 3 : 1 ratio (in terms of mass concentration). Details of the procedure are given in 4.4.3. The *Arabidopsis* mutant set consisted of *atpup1*, *atpup14*, *atent1*, *atent3*, *atent7*, *atent8*, and *atabcg14*; all of these muted genes had been previously studied in relation to cytokinin transport. As a negative control, wild-type *Arabidopsis* hypocotyls (ecotype Columbia, or Col-0) were treated in the same manner.

Results of 21-day long treatment are presented in Figure 14. All hypocotyls started forming shoot-like, green tissues. In a few cases, leaves sprouted from the hypocotyls as well. Despite a certain degree of diversity in the morphology of the tissues formed, no significant difference was observed between the control and either of the mutant explants.

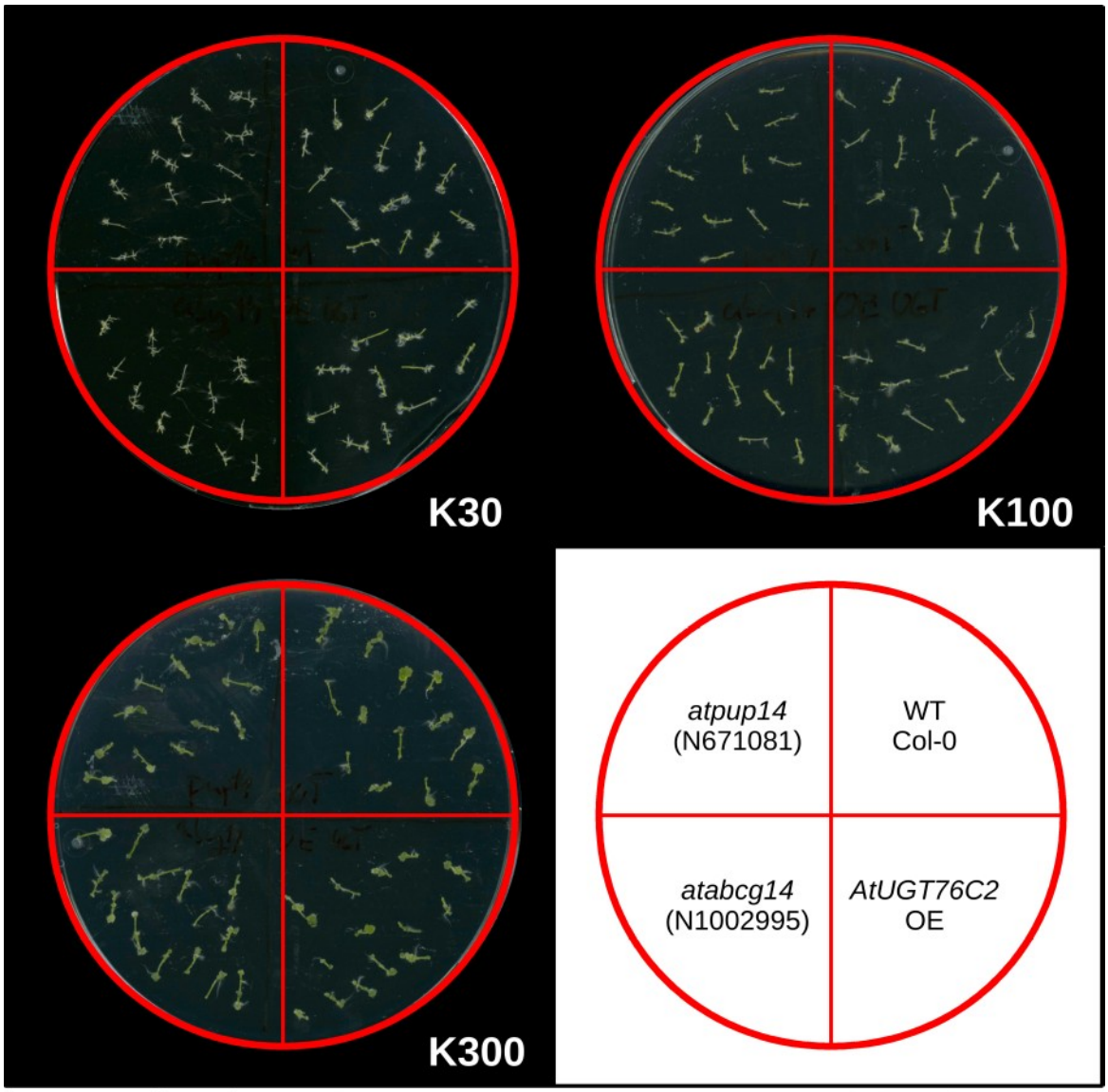

Figure 15: Effects of various kinetin concentration on organogenesis in Arabidopsis hypocotyl explants. The explants were cultivated on media containing 100 µg×L^{-1} auxin (1-naphthaleneacetic acid) and either 30 (K30), 100 (K100) or 300 µg×L^{-1} kinetin (K300). On each Petri dish, wild-type (WT; ecotype Columbia, or Col-0), AtUGT76C2-overexpressing (OE), atabcg14 and atpup14 hypocotyl explants were treated. The dishes were divided into four parts according to the scheme in the bottom right part of the figure. For mutant lines, NASC accession numbers are given in parentheses.

5.3.2 *De Novo* Tissue Formation in Wild-Type, *atabcg14*, *atpup14* and *AtUGT76C2*-Over-Expressing *Arabidopsis* Hypocotyls Treated With Various Cytokinins in Various Concentrations

To see how different cytokinin species and their different concentrations affect organogenetic processes in *Arabidopsis* hypocotyl explants, we concluded two more

experiments based on treating the hypocotyls with exogenous cytokinin and auxin. In both of them, we only focused on four types of hypocotyl explants: *atabcg14* and *atpup14* mutants, At*UGT76C2* overexpressor, and wild type.

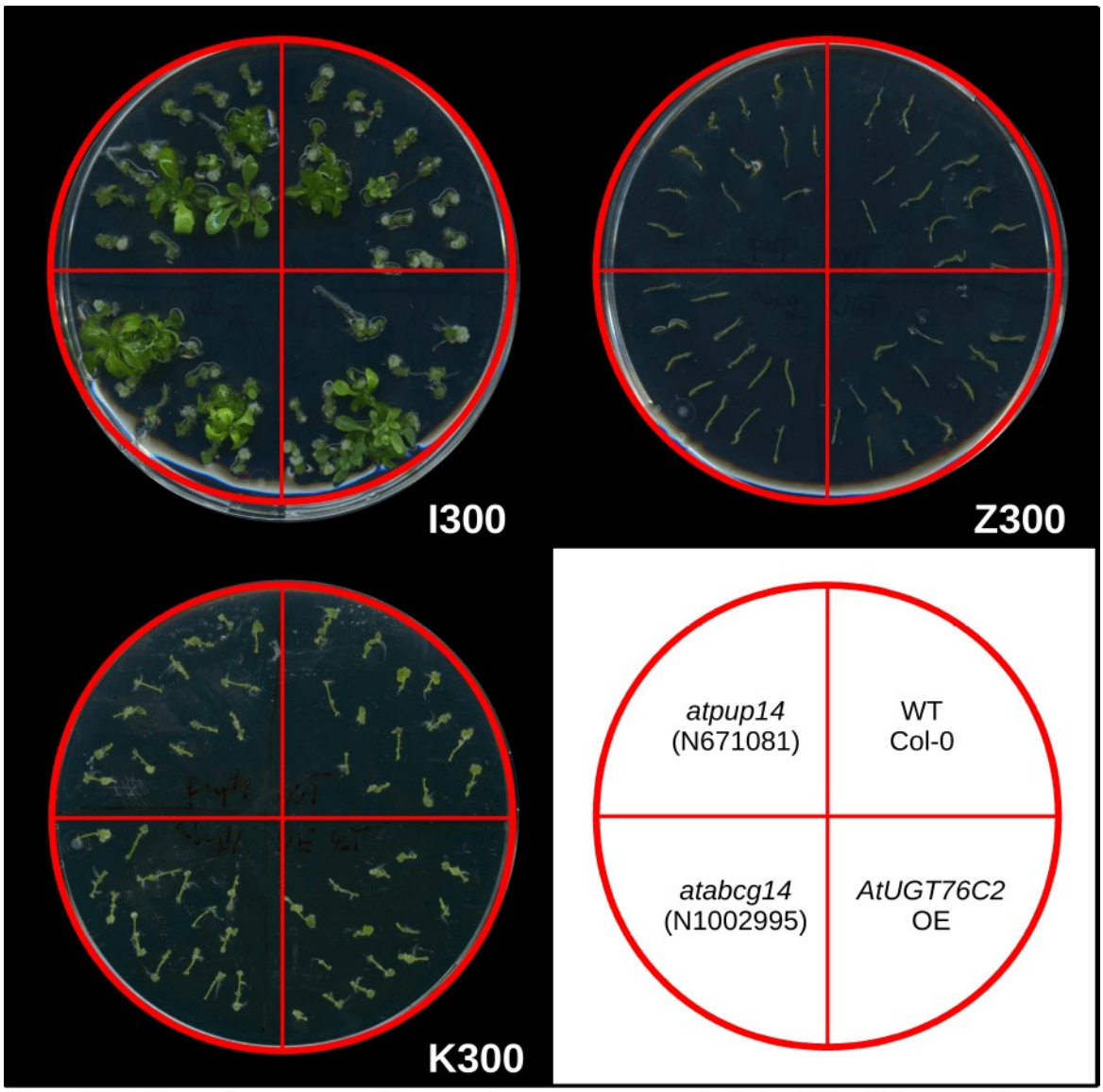

Figure 16: Effects of various cytokinin species on organogenesis in Arabidopsis hypocotyl explants. The explants were cultivated on media containing 100 µg×L^{-1} auxin (1-naphthaleneacetic acid) and 300 µg × L-1 cytokinin – either iP (I300), tZ (Z300) or kinetin (K300). On each Petri dish, wild-type (WT; ecotype Columbia, or Col-0), AtUGT76C2-overexpressing (OE), atabcg14 and atpup14 hypocotyl explants were treated. The dishes were divided into four parts according to the scheme in the bottom right part of the figure. For mutant lines, NASC accession numbers are given in parentheses.

In the first experiment, we compared the effects of different kinetin concentrations. Hypocotyl explants were treated by three different types of media, labelled as K30, K100 and K300. In the other experiment, we compared the effects of different cytokinin species. Hypocotyl explants were treated by media containing tZ (labelled Z300) and iP (labelled

P300) instead of kinetin. The results were compared with those previously obtained for K300. Cytokinin and auxin concentrations in each media type are given in Table 4.

Comparison of hypocotyl explant treatment with different kinetin concentrations is depicted in Figure 15. It can be seen that with increasing kinetin concentration (corresponding to the increasing number in media labels), the hypocotyls more readily differentiate into shoot-like tissues. When treated with K30 medium, most hypocotyls displayed growth of root-like structures, similar to those described in [219]. As the kinetin concentration increased, these structures diminished.

Comparison of hypocotyl explant treatment with kinetin, iP, and tZ at concentrations of 300 $\mu g \times L^{-1}$ (together with 100 $\mu g \times L^{-1}$ auxin) is depicted in Figure 16. Treating hypocotyl explants with iP resulted in a response corresponding to very high cytokinin doses. They differentiated into green calli, often sprouting shoot-like structures with leaves. On the other hand, hypocotyl explants treated with tZ displayed almost no callus growth. Their morphology corresponded to that of explants treated with kinetin at lower concentrations. The response of hypocotyl explants treated with kinetin was somewhere between those of explants treated with iP and tZ. There was no significant difference between responses of hypocotyl explants isolated from different plant types to either kind of hormonal treatment.

6 Discussion

6.1 Cytokinin Membrane Transport in Cell Suspension Cultures

6.1.1 Cytokinin N^9-Glucosides Are Not Transported Across The Plasma Membrane from Media to Tobacco BY-2 Cells

Transport processes on biological membranes are driven by the substrate's physically chemical and biological properties. Generally speaking, the simple diffusion is allowed to uncharged and hydrophobic molecule only and the carrier-mediated transport can be highly selective regarding the substrate's structure and role in the organism.

To study cytokinin transport on plasma membranes, we started by measuring accumulations of radio-labelled cytokinin traces in BY-2 cell suspension cultures during approximately fifteen minutes. We have observed a striking difference between uptakes of radio-labelled cytokinin free bases, [3H]-tZ and [3H]-iP, and their glycosylated counterparts, [3H]-tZ9G, [3H]-iP9G, and [3H]-cZ9G. While both free bases were readily accumulated by the cells, we detected only small and practically constant tracer amounts in cells accumulating cytokinin ribosides. These small values corresponded to those that were detected at the beginning of both [3H]-tZ and [3H]-iP accumulation assays. Therefore, it can be assumed that they originate in superficial contamination of the cells and that no actual transport of glycosylated cytokinins occurred.

It follows that conjugation with a glucose moiety is a change great enough to make cytokinins too polar to pass through biological membranes via the simple diffusion and unrecognisable for cytokinin membrane-bound transport systems. This is in agreement with their different roles, compared to cytokinin free bases, which act as biologically active chemical mediators. Cytokinin *O*-glucosides, which can be converted back to biologically active free bases via a β-glucosidase [96], and *N*-glucosides are usually described as storage and irreversibly inactivated cytokinin forms, respectively. However, *trans*-zeatin *O*-glucoside was also reported acting as a long-distance greening promoter in cucumber (*Cucumis sativus*) plants, distributed via xylem [227]. Furthermore, hydrolysis of cytokinin *N*-glucosides was observed too [93].

The authors in [228] state that in plants, glycosylation may serve to regulate compartmentalization of various compounds, which is in agreement with our findings that cytokinin bases and glucosides are not substrates of the same transport system.

The lack of any transport activity of cytokinin glucosides from media to BY-2 cells suggests that there is no need for plants to import such cytokinin forms from extracellular space into the cytoplasm. On the contrary, it has been hinted that cytokinin glucoside export from cells may occur *in planta*. Such a hypothesis is underlaid by different localization of most cytokinin glucosides and UGTs, catalysing glycosylation of cytokinin free bases. It has been shown that UGT85A1 and UGT76C2 localize to the cytoplasm; UGT76C1, bearing strong homology with the latter, is supposed to be a cytoplasmic enzyme as well [97], [229]. On the other hand, cytokinin glucosides were found mainly in extracellular space and vacuoles [230].

Based on these findings, the authors in [229] propose a general model of cytokinin action, in which cytokinin free bases, either formed in cells or imported from the extracellular space, are converted to glucosides in the cytosol and subsequently exported from cells or stored in vacuoles. The existence of such an export system would support the hypothesis that glucosides are not merely an inactive form of cytokinins, but that they rather play particular biological roles in plant organism. It remains to be answered whether the cytokinin glucosides distributed via xylem act as biologically active ligands of specific membrane receptors or whether are hydrolysed at the target tissues.

To further explore these possibilities, experiments focused on the putative cytokinin glucoside cellular export should be conducted. For instance, providing cell suspension cultures with radio-labelled cytokinin bases should result in the formation of radio-labelled cytokinin glucosides and their subsequent export back to media, where we should be able to detect them. The amount of exported cytokinin glucoside should be dependent on the initial tracer amount and also on the activities of cytokinin-specific UGTs.

6.1.2 *AtPUP14* Expression Has No Effect on Cytokinin Glucoside Transport from Media to Tobacco BY-2 Cells

Since no uptake of radio-labelled cytokinin glucosides occurred in wild-type BY-2 cell suspension cultures, we decided to see whether heterologous expression of *AtPUP14* gene, whose product was shown to act as a tZ-specific importer [155], changes this. However, no difference was observed between the cell lines expressing *AtPUP14* and those where the *AtPUP14* expression hadn't been induced, suggesting that glycosylation indeed prevents cytokinin to be recognised by membrane-bound transporters.

Interestingly, no change between induced and non-induced lines was observed in case of iP accumulation, either, even though iP had been shown to act as a tZ uptake

inhibitor in microsomal systems [155]. A possible explanation of such an observation is that there are membrane transport systems native to BY-2 cells with a significantly higher affinity toward iP than AtPUP14.

6.1.3 Kinetics of tZ and iP Uptake in BY-2 Cells Correspond to a Model Based on Michaelis-Menten Kinetics

In previously described experiments, we have shown that cytokinin free bases are readily transported across the plasma membrane from media to BY-2 cells. The kinetic studies have further shown that both tZ and iP uptake in BY-2 cells can be described using a model based on the classical Michaelis-Menten kinetics and competitive inhibition. It follows that these cytokinin free bases enter the cells both via carrier-mediated transport, which is dependent on the net capacity of all involved transporters (and therefore obeys saturation kinetics models), and simple diffusion, which is represented by the non-saturable component, as the diffusion rate depends only on the substrate concentration gradient.

Kinetics of tZ uptake in cell cultures were previously studied, for instance, by Cedzich et al. in [109]. The authors measured tZ uptake rate in *Arabidopsis* cells at various substrate concentration. They suggested the existence of high- and low-affinity tZ uptake systems. The former was studied at tZ concentrations ranging from 0 to 1000 nmol×L^{-1}, which is comparable to the settings we have used in this thesis. Cedzich et al. reported K_M of tZ uptake as 210.9 nmol×L^{-1} in *Arabidopsis* cells, while we have evaluated the same parameter in BY-2 cells as 108.3 nmol×L^{-1}. The fact that both these values lie within the order of 10^2 nmol×L^{-1} (even if they still somewhat differ) suggests similarities between tZ uptake in *Arabidopsis* and tobacco.

The low-affinity tZ uptake system, suggested by Cedzich et al., was studied at tZ concentrations ranging from 0 to 200 µmol×L^{-1}. Even though the transport rate depended on substrate concentration, it did not display the typical Michaelis-Menten saturation profile. The further mathematical analysis revealed this uptake system can be decomposed to at least two saturable functions, which are characterized by K_M values of 3.9 and 98.8 µmol×L^{-1}. The authors point out that the latter value is far from physiological cytokinin levels, which are typically nano-molar. Therefore, this lowest-affinity uptake may not be relevant *in planta* [109].

In the same work, tZ uptake inhibition was studied by adding various unlabelled competitors in ten-fold excess to the *Arabidopsis* cell suspension cultures. Addition of a ten-fold excess of iP lowered the tZ uptake rate by approximately 50 %, which the authors

interpreted as competition between the two cytokinin free bases [109]. In this thesis, we were able to support such a conclusion by measuring iP inhibitory effects on tZ uptake over a range of iP concentrations. The results could have been well fitted with a mathematical model based on competitive inhibition, expanded by the non-saturable component. When we had tried to measure tZ uptake inhibition by other cytokinin species, we got results we weren't able to fit anyhow (they are not shown in this thesis), which may serve as a confirmation that our model is sensitive enough to reject those cases where no competition occurs. In future research, we could broaden the results presented here by repeating the experiment with other substances previously reported as competitive tZ uptake inhibitors, such as adenine or 6-benzylaminopurine, and substances unrelated to cytokinins, such as allantoin [109], [155].

We have supposed that the inhibition between tZ and iP uptake in BY-2 cells is competitive, i.e. that both molecules bind to free transporters. In such cases, the presence of the inhibitor increases K_M of the substrate uptake, while other parameters, in our model V_{max} / ρ and NSR / ρ, remain unaffected. It is possible that either tZ or iP bind to transporter-substrate structures as well, therefore limiting the membrane transport systems capacity and lowering V_{max} / ρ. That would be a case of mixed inhibition, then. To explore this possibility, we could perform kinetic accumulation assays, as described in 4.4.7, with and without the inhibitor within the same cell suspension culture, to ensure that we work in a system with constant capacity.

In this thesis, we haven't further discriminated between facilitated diffusion and active transport, two types of carrier-mediated transport processes that differ in energy dependence. In plants, a large portion of secondary active transport processes is coupled to a proton gradient, maintained via proton pumps and production or consumption of protons during biochemical reactions [231]–[233]. Therefore, the energy dependence of membrane transport processes is often tested by addition of a protonophore, such as CCCP, which is supposed to halt the transport via cancelling the proton gradient [109], [215], [234].

6.2 Cytokinin Metabolism in *Arabidopsis* Plants

6.2.1 Mutations of *atabcg14* and *atpup14* Altered Net Uptake of Radio-Labelled Cytokinin Free Bases in *Arabidopsis* Plants

To examine the effects of *atabcg14* and *atpup14* mutations, we treated 14-day old *Arabidopsis* plants with MS/2 media containing radio-labelled cytokinin free bases and

glucosides in concentrations of 20 nmol×L^{-1}. Having analysed media samples taken before and after a 100-minute plant treatment, we observed that little or no uptake of the glucosides occurred. This agrees with the result obtained from accumulation assays in BY-2 cells, indicating that cytokinin glycosides do not readily enter plant cells via the cytoplasmic membrane.

The results obtained for treating *Arabidopsis* plants with radio-labelled cytokinin glucosides are presented, but their accuracy is questionable. In future, to study the cytokinin glucosides metabolism, other methods than their exogenous application should be employed, such as cytokinin profiling in glucoside-over-expressing plants.

On the other hand, some uptake by *Arabidopsis* plants was observed in case of both radio-labelled cytokinin free bases, [3H]-tZ and [3H]-iP. Both types of mutant plants, *atabcg14* and *atpup14*, were impaired in terms of cytokinin free bases net uptake, which could be caused by both reduced import of radio-labelled tracers from media to plants and increased export of radio-labelled metabolites from plants to media. To be able to discuss both these effects, cytokinin profiling in the plants and the media samples was performed.

6.2.2 Mutations of *atabcg14* and *atpup14* Altered Cytokinin Free Bases Metabolism

Metabolic profiles of *atpup14* plants display a reduced amount of tZ in plant tissues. On the other hand, the relative amount of adenine in plant tissues, when compared to wild-type plants, increased. What's more, in media used to treat *atpup14* plants with [3H]-tZ, the relative amount of adenine significantly grew during the 100-minute long treatment, while the relative amount of tZ in the same media significantly dropped. If the *atpup14* mutation had simply reduced tZ uptake by the transgenic plants, we would obtain opposite results – not to mention that a single mutation is unlikely to impair cytokinin uptake by the whole plants.

A more plausible interpretation is that while tZ uptake by *atpup14* plants remained primarily unaffected, tZ import to cells was hindered, which caused tZ to accumulate in the apoplast. There, it became substrate of extracellular CKX isoforms, which are specific toward cytokinin free bases, unlike those localized in cellular compartments [235], [236]. The CKXs thus converted tZ to adenine more readily in *atpup14* plants than in the wild-type ones. Moreover, because of the hindered tZ transport to cells, a smaller portion of the radio-labelled tZ could have been converted to its conjugated forms, which would have provided a certain degree of protection from the apoplastic CKXs. Such a statement can be

supported by the fact that relative amounts of tZ7G, tZR, and tZRMP were smaller in *atpup14* plants than in the wild-type ones. Adenine, the product of tZ degradation, could have been eventually exported back to media.

The increased rate of tZ degradation, followed by an increased adenine export to media, would explain all the observations mentioned above - the decreased drop of the total radioactivity, the decreased amount of tZ in plant tissues, and the increased amount of adenine in both plant tissues and media samples taken after the treatment in *atpup14* plants, all in comparison to the wild-type ones. This interpretation also supports the view of AtPUP14 as a specific transporter of cytokinin free bases from apoplast to the cytoplasm [155].

Similar trends can be seen in the results obtained after the *atpup14* plants were treated with radio-labelled iP, even though the differences between *atpup14* and wild-type plants were smaller. Supposing that these differences stem from the impaired iP cellular import, the less pronounced effects of the *atpup14* mutation on iP metabolic profiles, in comparison to tZ, might be a net result of AtPUP14 displaying lower affinity to iP, lower uptake of iP by *Arabidopsis* plants, and higher iP degradation rate.

The results of metabolic profiling in *atabcg14* plants were similar to those observed in the case of *atpup14* plants and discussed above. The tZ relative content in *atabcg14* plant tissues wasn't much different from that in wild-type plants, but the respective increase and decrease of adenine and tZ relative amounts, previously discussed in relation to *atpup14*, were observed in media used to treat *atabcg14* plants with radio-labelled tZ too. Since AtABCG14 has been described as cytokinin-specific exporter localized to root tissues [157], [159], another explanation of the observed phenomena should be devised. The increase of adenine relative content in media used to treat *atabcg14* plants may be relevant to tZ or iP accumulation in root cells, given their impaired export to xylem because of the *atabcg14* mutation. To maintain cytokinin homoeostasis, the plants might have converted the surplus tZ and iP to adenine, which was subsequently exported back to media.

Another two points should be noted about our results. Firstly, the plants were submerged during the experiment. Such a situation, far from *Arabidopsis* natural living conditions, might have triggered a stress response changing some metabolic processes. And secondly, both mutations might have caused secondary defects in the plants, affecting their behaviour during our experiments.

6.3 Cytokinin-Mediated Organogenesis in *Arabidopsis* Hypocotyl Explants

6.3.1 Phenotypes of *atabcg14*, *atpup14*, and *AtUGT76C2*-Over-Expressing *Arabidopsis* Hypocotyl Explants Were Not Different From The Wild Type

We were interested to see if hypocotyl explant assays described in [219], [220] can help us to further characterize the roles of *AtPUP14* and *AtABCG14* genes in cytokinin transport. We saw no effects of either mutation on cytokinin-treated *Arabidopsis* hypocotyl explant phenotypes. Similarly, no differences in cytokinin-induced organogenesis were observed between the wild-type explants and those over-expressing *UGT76C2*, which we included for further comparison. On the other hand, the phenotypes varied if the hypocotyl explants were treated with different cytokinin species or with different concentrations of the same cytokinin. Neither of the mutations, nor *UGT76C2* over-expression is a change significant enough to alter *de novo* organogenesis.

We concluded that both *AtABCG14* and *AtPUP14* become important only in tissues at certain developmental stages, while in hypocotyls and hypocotyl-based calli, long-distance transport and simple diffusion dominate, making the roles of both transporter genes insignificant. This corresponds with the specific expression patterns of both genes [155], [157], [159].

7 Conclusions

1. In BY-2 cell suspension culture, tZ and iP uptake were observed. On the other hand, no uptake of tZ9G, iP9G, or cZ9G occurred.
2. Carrier-mediated transport of cytokinin free bases across cytoplasmic membrane in BY-2 cell suspension culture was confirmed. K_M values of tZ and iP uptake in BY-2 cells were approximately evaluated as 108 and 65 nmol × L^{-1}, respectively. Competitive inhibition between the two cytokinins was observed.
3. In 14-day old *Arabidopsis* plants treated with MS/2 media containing radio-labelled cytokinins, tZ and iP uptake was observed. In *atabcg14* and *atpup14* mutants, the difference between total radioactivity in media samples taken before and after the treatment varied from the difference detected in wild-type plants.
4. In both *atabcg14* and *atpup14* mutants, increase in tZ and iP conversion to adenine and its subsequent secretion to media were observed. In *atpup14* mutants, the relative amount of tZ in plant tissues was lower than in wild-type plants.
5. In *Arabidopsis* hypocotyl explants, no phenotype response was triggered by either *atabcg14* mutation, *atpup14* mutation, and *AtUGT76C2* over-expression.

8 References

[1] J. D. B. Weyers and N. W. Paterson, ‘Plant hormones and the control of physiological processes’, *New Phytologist*, vol. 152, no. 3, pp. 375–407, 2001, doi: 10.1046/j.0028-646X.2001.00281.x.

[2] H. B. Tukey, F. W. Went, R. M. Muir, and J. V. Overbreek, ‘Nomenclature of Chemical Plant Regulators’, *Plant Physiol*, vol. 29, no. 3, pp. 307–308, May 1954.

[3] C. Darwin and F. Darwin, *Power of Movement in Plants*. John Murray, 1880.

[4] M. Estelle, ‘Plant tropisms: The ins and outs of auxin’, *Current Biology*, vol. 6, no. 12, pp. 1589–1591, Dec. 1996, doi: 10.1016/S0960-9822(02)70780-X.

[5] C. W. Whippo and R. P. Hangarter, ‘Phototropism: Bending towards Enlightenment’, *The Plant Cell*, vol. 18, no. 5, pp. 1110–1119, May 2006, doi: 10.1105/tpc.105.039669.

[6] K. V. Thimann and J. B. Koepfli, ‘Identity of the Growth-Promoting and Root-Forming Substances of Plants’, *Nature*, vol. 135, no. 3403, pp. 101–102, Jan. 1935, doi: 10.1038/135101a0.

[7] D. Weijers, J. Nemhauser, and Z. Yang, ‘Auxin: small molecule, big impact’, *J Exp Bot*, vol. 69, no. 2, pp. 133–136, Jan. 2018, doi: 10.1093/jxb/erx463.

[8] J.-M. Davière and P. Achard, ‘Gibberellin signaling in plants’, *Development*, vol. 140, no. 6, pp. 1147–1151, Mar. 2013, doi: 10.1242/dev.087650.

[9] T. YABUTA, ‘On the crystal of gibberellin, a substance to promote plant growth’, *J. Agric. Chem. Soc. Japan*, vol. 14, p. 1526, 1938.

[10] S. D. Clouse, ‘Brassinosteroids’, *Arabidopsis Book*, vol. 9, Nov. 2011, doi: 10.1199/tab.0151.

[11] J. W. Mitchell, N. Mandava, J. F. Worley, J. R. Plimmer, and M. V. Smith, ‘Brassins--a new family of plant hormones from rape pollen’, *Nature*, vol. 225, no. 5237, pp. 1065–1066, Mar. 1970, doi: 10.1038/2251065a0.

[12] C. E. Cook, L. P. Whichard, B. Turner, M. E. Wall, and G. H. Egley, ‘Germination of Witchweed (Striga lutea Lour.): Isolation and Properties of a Potent Stimulant’, *Science*, vol. 154, no. 3753, pp. 1189–1190, Dec. 1966, doi: 10.1126/science.154.3753.1189.

[13] B. Zwanenburg, T. Pospíšil, and S. Ćavar Zeljković, ‘Strigolactones: new plant hormones in action’, *Planta*, vol. 243, no. 6, pp. 1311–1326, Jun. 2016, doi: 10.1007/s00425-015-2455-5.

[14] A. Bakshi, J. M. Shemansky, C. Chang, and B. M. Binder, ‘History of Research on the Plant Hormone Ethylene’, *J Plant Growth Regul*, vol. 34, no. 4, pp. 809–827, Dec. 2015, doi: 10.1007/s00344-015-9522-9.

[15] Y. Boursiac, S. Léran, C. Corratgé-Faillie, A. Gojon, G. Krouk, and B. Lacombe, ‘ABA transport and transporters’, *Trends in Plant Science*, vol. 18, no. 6, pp. 325–333, Jun. 2013, doi: 10.1016/j.tplants.2013.01.007.

[16] W.-C. Liu and H. R. Carnsdagger, ‘Isolation of Abscisin, an Abscission Accelerating Substance’, *Science*, vol. 134, no. 3476, pp. 384–385, Aug. 1961, doi: 10.1126/science.134.3476.384.

[17] E. Demole, E. Lederer, and D. Mercier, ‘Isolement et détermination de la structure du jasmonate de méthyle, constituant odorant caractéristique de l’essence de jasmin’, *Helvetica Chimica Acta*, vol. 45, no. 2, pp. 675–685, 1962, doi: 10.1002/hlca.19620450233.

[18] C. Wasternack, ‘Action of jasmonates in plant stress responses and development — Applied aspects’, *Biotechnology Advances*, vol. 32, no. 1, pp. 31–39, Jan. 2014, doi: 10.1016/j.biotechadv.2013.09.009.

[19] D. A. Dempsey and D. F. Klessig, ‘How does the multifaceted plant hormone salicylic acid combat disease in plants and are similar mechanisms utilized in humans?’, *BMC Biol*, vol. 15, Mar. 2017, doi: 10.1186/s12915-017-0364-8.

[20] I. Raskin, I. M. Turner, and W. R. Melander, ‘Regulation of heat production in the inflorescences of an Arum lily by endogenous salicylic acid’, *Proc. Natl. Acad. Sci. U.S.A.*, vol. 86, no. 7, pp. 2214–2218, Apr. 1989, doi: 10.1073/pnas.86.7.2214.

[21] C. A. Ryan and G. Pearce, ‘Polypeptide Hormones’, *Plant Physiology*, vol. 125, no. 1, pp. 65–68, Jan. 2001, doi: 10.1104/pp.125.1.65.

[22] P. J. Davies, ‘The Plant Hormones: Their Nature, Occurrence, and Functions’, in *Plant Hormones*, P. J. Davies, Ed. Dordrecht: Springer Netherlands, 2010, pp. 1–15.

[23] E. Ricciotti and G. A. FitzGerald, ‘Prostaglandins and Inflammation’, *Arterioscler Thromb Vasc Biol*, vol. 31, no. 5, pp. 986–1000, May 2011, doi: 10.1161/ATVBAHA.110.207449.

[24] Y. Zhao, ‘Auxin Biosynthesis: A Simple Two-Step Pathway Converts Tryptophan to Indole-3-Acetic Acid in Plants’, *Mol Plant*, vol. 5, no. 2, pp. 334–338, Mar. 2012, doi: 10.1093/mp/ssr104.

[25] A. Chattoraj, T. Liu, L. S. Zhang, Z. Huang, and J. Borjigin, ‘Melatonin formation in mammals: In vivo perspectives’, *Rev Endocr Metab Disord*, vol. 10, no. 4, pp. 237–243, Dec. 2009, doi: 10.1007/s11154-009-9125-5.
[26] Y. H. Wang and H. R. Irving, ‘Developing a model of plant hormone interactions’, *Plant Signal Behav*, vol. 6, no. 4, pp. 494–500, Apr. 2011, doi: 10.4161/psb.6.4.14558.
[27] G. E. Schaller, A. Bishopp, and J. J. Kieber, ‘The Yin-Yang of Hormones: Cytokinin and Auxin Interactions in Plant Development’, *The Plant Cell Online*, vol. 27, no. 1, pp. 44–63, Jan. 2015, doi: 10.1105/tpc.114.133595.
[28] J. Pavlů, J. Novák, V. Koukalová, M. Luklová, B. Brzobohatý, and M. Černý, ‘Cytokinin at the Crossroads of Abiotic Stress Signalling Pathways’, *International Journal of Molecular Sciences*, vol. 19, no. 8, p. 2450, Aug. 2018, doi: 10.3390/ijms19082450.
[29] H. Rouached, A. B. Arpat, and Y. Poirier, ‘Regulation of Phosphate Starvation Responses in Plants: Signaling Players and Cross-Talks’, *Molecular Plant*, vol. 3, no. 2, pp. 288–299, Mar. 2010, doi: 10.1093/mp/ssp120.
[30] W. S. Pierpoint, ‘The natural history of salicylic acid Plant product and mammalian medicine’, *Interdisciplinary Science Reviews*, Jul. 2013, doi: 10.1179/isr.1997.22.1.45.
[31] A. G. Pirbalouti, S. E. Sajjadi, and K. Parang, ‘A Review (Research and Patents) on Jasmonic Acid and Its Derivatives’, *Archiv der Pharmazie*, vol. 347, no. 4, pp. 229–239, 2014, doi: 10.1002/ardp.201300287.
[32] E. M. Othman, M. Naseem, E. Awad, T. Dandekar, and H. Stopper, ‘The Plant Hormone Cytokinin Confers Protection against Oxidative Stress in Mammalian Cells’, *PLoS One*, vol. 11, no. 12, Dec. 2016, doi: 10.1371/journal.pone.0168386.
[33] H. Cronin and Z. D. Draelos, ‘Original Contribution: Top 10 botanical ingredients in 2010 anti-aging creams’, *Journal of Cosmetic Dermatology*, vol. 9, no. 3, pp. 218–225, 2010, doi: 10.1111/j.1473-2165.2010.00516.x.
[34] K. Grossmann, ‘Auxin Herbicide Action’, *Plant Signal Behav*, vol. 2, no. 5, pp. 421–423, 2007.
[35] S. D. T. Maduwanthi and R. A. U. J. Marapana, ‘Induced Ripening Agents and Their Effect on Fruit Quality of Banana’, *Int J Food Sci*, vol. 2019, May 2019, doi: 10.1155/2019/2520179.
[36] T. Oono, ‘Production of Seedless Grapes by Gibberellin Treatment’, *Japan Agricultural Research Quarterly*, vol. 7, no. 1, pp. 35–37, Jan. 1973.
[37] T. Pandolfini, ‘Seedless Fruit Production by Hormonal Regulation of Fruit Set’, *Nutrients*, vol. 1, no. 2, pp. 168–177, Nov. 2009, doi: 10.3390/nu1020168.
[38] T. Werner and T. Schmülling, ‘Cytokinin action in plant development’, *Current Opinion in Plant Biology*, vol. 12, no. 5, pp. 527–538, Oct. 2009, doi: 10.1016/j.pbi.2009.07.002.
[39] L. Spíchal, ‘Cytokinins - recent news and views of evolutionally old molecules’, *Functional Plant Biology*, vol. 39, no. 4, p. 267, 2012, doi: 10.1071/FP11276.
[40] J. J. Kieber and G. E. Schaller, ‘Cytokinins’, *The Arabidopsis Book*, vol. 12, p. e0168, Jan. 2014, doi: 10.1199/tab.0168.
[41] D. S. Letham, ‘Zeatin, a factor inducing cell division isolated from Zea mays’, *Life Sciences*, vol. 2, no. 8, pp. 569–573, Aug. 1963, doi: 10.1016/0024-3205(63)90108-5.
[42] D. S. Letham, ‘Cytokinins from Zea mays’, *Phytochemistry*, vol. 12, no. 10, pp. 2445–2455, Oct. 1973, doi: 10.1016/0031-9422(73)80453-4.
[43] S. Gajdošová *et al.*, ‘Distribution, biological activities, metabolism, and the conceivable function of cis-zeatin-type cytokinins in plants’, *Journal of Experimental Botany*, vol. 62, no. 8, pp. 2827–2840, May 2011, doi: 10.1093/jxb/erq457.
[44] A. Heyl, M. Riefler, G. A. Romanov, and T. Schmülling, ‘Properties, functions and evolution of cytokinin receptors’, *European Journal of Cell Biology*, vol. 91, no. 4, pp. 246–256, Apr. 2012, doi: 10.1016/j.ejcb.2011.02.009.
[45] M. Strnad *et al.*, ‘Meta-topolin, a highly active aromatic cytokinin from poplar leaves (Populus × canadensis Moench., cv. Robusta)’, *Phytochemistry*, vol. 45, no. 2, pp. 213–218, May 1997, doi: 10.1016/S0031-9422(96)00816-3.
[46] M. Strnad, ‘The aromatic cytokinins’, *Physiologia Plantarum*, vol. 101, no. 4, pp. 674–688, Dec. 1997, doi: 10.1111/J.1399-3054.1997.TB01052.X.
[47] D. Tarkowská *et al.*, ‘Identification of new aromatic cytokinins in *Arabidopsis thaliana* and *Populus x canadensis* leaves by LC-(+)ESI-MS and capillary liquid chromatography/frit–fast atom bombardment mass spectrometry’, *Physiologia Plantarum*, vol. 117, no. 4, pp. 579–590, Apr. 2003, doi: 10.1034/j.1399-3054.2003.00071.x.

[48] C. O. Miller, F. Skoog, F. S. Okumura, M. H. von Saltza, and F. M. Strong, ‘Structure and Synthesis of Kinetin’, *J. Am. Chem. Soc.*, vol. 77, no. 9, pp. 2662–2663, 1955, doi: 10.1021/ja01614a108.
[49] C. O. Miller, F. Skoog, F. S. Okumura, M. H. von Saltza, and F. M. Strong, ‘Isolation, Structure and Synthesis of Kinetin, a Substance Promoting Cell Division’, *J. Am. Chem. Soc.*, vol. 78, no. 7, pp. 1375–1380, 1956, doi: 10.1021/ja01588a032.
[50] C. O. Miller, F. Skoog, M. H. von Saltza, and F. M. Strong, ‘Kinetin, a Cell Division Factor From Deoxyribonucleic Acid’, *J. Am. Chem. Soc.*, vol. 77, no. 5, p. 1392, 1955, doi: 10.1021/ja01610a105.
[51] H. Yamada *et al.*, ‘The Arabidopsis AHK4 Histidine Kinase is a Cytokinin-Binding Receptor that Transduces Cytokinin Signals Across the Membrane’, *Plant Cell Physiol*, vol. 42, no. 9, pp. 1017–1023, Sep. 2001, doi: 10.1093/pcp/pce127.
[52] M. C. Mok *et al.*, ‘Topolins and Hydroxylated Thidiazuron Derivatives Are Substrates of Cytokinin O-Glucosyltransferase with Position Specificity Related to Receptor Recognition’, *Plant Physiol*, vol. 137, no. 3, pp. 1057–1066, Mar. 2005, doi: 10.1104/pp.104.057174.
[53] L. Spíchal *et al.*, ‘Two Cytokinin Receptors of Arabidopsis thaliana, CRE1/AHK4 and AHK3, Differ in their Ligand Specificity in a Bacterial Assay’, *Plant Cell Physiol*, vol. 45, no. 9, pp. 1299–1305, Sep. 2004, doi: 10.1093/pcp/pch132.
[54] R. J. Jones and B. M. N. Schreiber, ‘Role and function of cytokinin oxidase in plants’, *Plant Growth Regulation*, vol. 23, no. 1, pp. 123–134, Oct. 1997, doi: 10.1023/A:1005913311266.
[55] B. C. Persson, B. Esberg, Ó. Ólafsson, and G. R. Björk, ‘Synthesis and function of isopentenyl adenosine derivatives in tRNA’, *Biochimie*, vol. 76, no. 12, pp. 1152–1160, Jan. 1994, doi: 10.1016/0300-9084(94)90044-2.
[56] A. Golovko, G. Hjälm, F. Sitbon, and B. Nicander, ‘Cloning of a human tRNA isopentenyl transferase’, *Gene*, vol. 258, no. 1, pp. 85–93, Nov. 2000, doi: 10.1016/S0378-1119(00)00421-2.
[57] I. Hwang and H. Sakakibara, ‘Cytokinin biosynthesis and perception’, *Physiologia Plantarum*, vol. 126, no. 4, pp. 528–538, 2006, doi: 10.1111/j.1399-3054.2006.00665.x.
[58] B. M. Lange, T. Rujan, W. Martin, and R. Croteau, ‘Isoprenoid biosynthesis: The evolution of two ancient and distinct pathways across genomes’, *PNAS*, vol. 97, no. 24, pp. 13172–13177, Nov. 2000, doi: 10.1073/pnas.240454797.
[59] M. Rohmer, ‘The discovery of a mevalonate-independent pathway for isoprenoid biosynthesis in bacteria, algae and higher plants’, *Nat. Prod. Rep.*, vol. 16, no. 5, pp. 565–574, 1999, doi: 10.1039/a709175c.
[60] A. Banerjee and T. D. Sharkey, ‘Methylerythritol 4-phosphate (MEP) pathway metabolic regulation’, *Nat. Prod. Rep.*, vol. 31, no. 8, pp. 1043–1055, Jul. 2014, doi: 10.1039/C3NP70124G.
[61] S. Hecht *et al.*, ‘Studies on the nonmevalonate pathway to terpenes: The role of the GcpE (IspG) protein’, *PNAS*, vol. 98, no. 26, pp. 14837–14842, Dec. 2001, doi: 10.1073/pnas.201399298.
[62] Y. Taya, Y. Tanaka, and S. Nishimura, ‘5′-AMP is a direct precursor of cytokinin in Dictyostelium discoideum’, *Nature*, vol. 271, no. 5645, pp. 545–547, Feb. 1978, doi: 10.1038/271545a0.
[63] D. E. Akiyoshi, H. Klee, R. M. Amasino, E. W. Nester, and M. P. Gordon, ‘T-DNA of Agrobacterium tumefaciens encodes an enzyme of cytokinin biosynthesis’, *PNAS*, vol. 81, no. 19, pp. 5994–5998, Oct. 1984, doi: 10.1073/pnas.81.19.5994.
[64] K. Takei, H. Sakakibara, and T. Sugiyama, ‘Identification of Genes Encoding Adenylate Isopentenyltransferase, a Cytokinin Biosynthesis Enzyme, in Arabidopsis thaliana’, *J. Biol. Chem.*, vol. 276, no. 28, pp. 26405–26410, Jul. 2001, doi: 10.1074/jbc.M102130200.
[65] T. Kakimoto, ‘Identification of Plant Cytokinin Biosynthetic Enzymes as Dimethylallyl Diphosphate:ATP/ADP Isopentenyltransferases’, *Plant and Cell Physiology*, vol. 42, no. 7, pp. 677–685, Jul. 2001, doi: 10.1093/pcp/pce112.
[66] H. Kasahara *et al.*, ‘Distinct Isoprenoid Origins of cis- and trans-Zeatin Biosyntheses in Arabidopsis’, *J. Biol. Chem.*, vol. 279, no. 14, pp. 14049–14054, Feb. 2004, doi: 10.1074/jbc.M314195200.
[67] A. Golovko, F. Sitbon, E. Tillberg, and B. Nicander, ‘Identification of a tRNA isopentenyltransferase gene from Arabidopsis thaliana’, *Plant Mol Biol*, vol. 49, no. 2, pp. 161–169, May 2002, doi: 10.1023/A:1014958816241.
[68] K. Miyawaki *et al.*, ‘Roles of Arabidopsis ATP/ADP isopentenyltransferases and tRNA isopentenyltransferases in cytokinin biosynthesis.’, *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 44, pp. 16598–603, Oct. 2006, doi: 10.1073/pnas.0603522103.
[69] H. J. Vreman, R. Thomas, J. Corse, S. Swaminathan, and N. Murai, ‘Cytokinins in tRNA Obtained from Spinacia oleracea L. Leaves and Isolated Chloroplasts’, *Plant Physiology*, vol. 61, no. 2, pp. 296–306, Feb. 1978, doi: 10.1104/pp.61.2.296.

[70] K. Takei, T. Yamaya, and H. Sakakibara, ‘Arabidopsis CYP735A1 and CYP735A2 encode cytokinin hydroxylases that catalyze the biosynthesis of trans-Zeatin.’, *The Journal of biological chemistry*, vol. 279, no. 40, pp. 41866–72, Oct. 2004, doi: 10.1074/jbc.M406337200.

[71] S. B. Gelvin, ‘Agrobacterium-Mediated Plant Transformation: the Biology behind the “Gene-Jockeying” Tool’, *Microbiol Mol Biol Rev*, vol. 67, no. 1, pp. 16–37, Mar. 2003, doi: 10.1128/MMBR.67.1.16-37.2003.

[72] H. Sakakibara *et al.*, ‘Agrobacterium tumefaciens increases cytokinin production in plastids by modifying the biosynthetic pathway in the host plant’, *PNAS*, vol. 102, no. 28, pp. 9972–9977, 2005, doi: 10.1073/pnas.0500793102.

[73] C.-M. Chen and S. M. Kristopeit, ‘Metabolism of Cytokinin: Dephosphorylation of Cytokinin Ribonucleotide by 5’-Nucleotidases From Wheat Germ Cytosol’, *Plant physiology*, vol. 67, no. 3, pp. 494–8, Mar. 1981, doi: 10.1104/PP.67.3.494.

[74] C.-M. Chen and S. M. Kristopeit, ‘Metabolism of Cytokinin: Deribosylation of Cytokinin Ribonucleoside by Adenosine Nucleosidase from Wheat Germ Cells’, *Plant Physiol*, vol. 68, pp. 1020–1023, 1981.

[75] T. Kurakawa *et al.*, ‘Direct control of shoot meristem activity by a cytokinin-activating enzyme’, *Nature*, vol. 445, pp. 652–655, Feb. 2007, doi: 10.1038/nature05504.

[76] T. Kuroha *et al.*, ‘Functional analyses of LONELY GUY cytokinin-activating enzymes reveal the importance of the direct activation pathway in Arabidopsis.’, *The Plant cell*, vol. 21, no. 10, pp. 3152–69, Oct. 2009, doi: 10.1105/tpc.109.068676.

[77] H. Tokunaga *et al.*, ‘Arabidopsis lonely guy (LOG) multiple mutants reveal a central role of the LOG-dependent pathway in cytokinin activation’, *The Plant Journal*, vol. 69, no. 2, pp. 355–365, 2012, doi: 10.1111/j.1365-313X.2011.04795.x.

[78] R. C. Martin, M. C. Mok, G. Shaw, and D. W. S. Mok, ‘An Enzyme Mediating the Conversion of Zeatin to Dihydrozeatin in Phaseolus Embryos 1’, *Plant Physiol*, vol. 90, no. 4, pp. 1630–1635, Aug. 1989.

[79] N. V. Bassil, D. W. S. Mok, and M. C. Mok, ‘Partial Purification of a cis-trans-Isomerase of Zeatin from Immature Seed of Phaseolus vulgaris L’, *Plant Physiol.*, vol. 102, no. 3, pp. 867–872, Jul. 1993.

[80] T. Hluska, M. Šebela, R. Lenobel, I. Frébort, and P. Galuszka, ‘Purification of Maize Nucleotide Pyrophosphatase/Phosphodiesterase Casts Doubt on the Existence of Zeatin Cis–Trans Isomerase in Plants’, *Front. Plant Sci.*, vol. 8, 2017, doi: 10.3389/fpls.2017.01473.

[81] V. Pačes, E. Werstiuk, and R. H. Hall, ‘Conversion of N6-(A2-Isopentenyl)adenosine to Adenosine by Enzyme Activity in Tobacco Tissue’, *Plant Physiol*, vol. 48, pp. 775–778, 1971.

[82] P. D. Hare and J. Staden, ‘Cytokinin oxidase: Biochemical features and physiological significance’, *Physiologia Plantarum*, vol. 91, no. 1, pp. 128–136, May 1994, doi: 10.1111/j.1399-3054.1994.tb00668.x.

[83] N. Houba-Hérin, C. Pethe, J. D’Alayer, and M. Laloue, ‘Cytokinin oxidase from Zea mays: purification, cDNA cloning and expression in moss protoplasts’, *The Plant Journal*, vol. 17, no. 6, pp. 615–626, 1999, doi: 10.1046/j.1365-313X.1999.00408.x.

[84] R. O. Morris, K. D. Bilyeu, J. G. Laskey, and N. N. Cheikh, ‘Isolation of a Gene Encoding a Glycosylated Cytokinin Oxidase from Maize’, *Biochemical and Biophysical Research Communications*, vol. 255, no. 2, pp. 328–333, Feb. 1999, doi: 10.1006/bbrc.1999.0199.

[85] T. Schmülling, T. Werner, M. Riefler, E. Krupková, and I. Bartrina y Manns, ‘Structure and function of cytokinin oxidase/dehydrogenase genes of maize, rice, Arabidopsis and other species’, *Journal of Plant Research*, vol. 116, no. 3, pp. 241–252, Jun. 2003, doi: 10.1007/s10265-003-0096-4.

[86] T. Werner, V. Motyka, M. Strnad, and T. Schmülling, ‘Regulation of plant growth by cytokinin’, *PNAS*, vol. 98, no. 18, pp. 10487–10492, Aug. 2001, doi: 10.1073/pnas.171304098.

[87] K. D. Bilyeu *et al.*, ‘Molecular and Biochemical Characterization of a Cytokinin Oxidase from Maize’, *Plant Physiol*, vol. 125, no. 1, pp. 378–386, Jan. 2001.

[88] P. Galuszka, I. Frébort, M. Šebela, P. Sauer, S. Jacobsen, and P. Peč, ‘Cytokinin oxidase or dehydrogenase?’, *European Journal of Biochemistry*, vol. 268, no. 2, pp. 450–461, Jan. 2001, doi: 10.1046/j.1432-1033.2001.01910.x.

[89] J. M. Chatfield and D. J. Armstrong, ‘Cytokinin Oxidase from Phaseolus vulgaris Callus Tissues : Enhanced in Vitro Activity of the Enzyme in the Presence of Copper-Imidazole Complexes’, *Plant Physiology*, vol. 84, no. 3, pp. 726–731, Jul. 1987, doi: 10.1104/pp.84.3.726.

[90] P. Galuszka *et al.*, ‘Cytokinins as Inhibitors of Plant Amine Oxidase’, *Journal of Enzyme Inhibition*, vol. 13, no. 6, pp. 457–463, Jan. 1998, doi: 10.3109/14756369809020549.

[91] K. Yonekura-Sakakibara and K. Hanada, ‘An evolutionary view of functional diversity in family 1 glycosyltransferases’, *The Plant Journal*, vol. 66, no. 1, pp. 182–193, 2011, doi: 10.1111/j.1365-313X.2011.04493.x.

[92] B. Hou, E.-K. Lim, G. S. Higgins, and D. J. Bowles, ‘N-Glucosylation of Cytokinins by Glycosyltransferases of Arabidopsis thaliana’, *J. Biol. Chem.*, vol. 279, no. 46, pp. 47822–47832, Dec. 2004, doi: 10.1074/jbc.M409569200.

[93] P. Hošek *et al.*, ‘Distinct metabolism of N-glucosides of isopentenyladenine and trans-zeatin determines cytokinin metabolic spectrum in Arabidopsis’, *New Phytologist*, vol. 225, no. 6, pp. 2423–2438, 2020, doi: 10.1111/nph.16310.

[94] J. Wang, X.-M. Ma, M. Kojima, H. Sakakibara, and B.-K. Hou, ‘Glucosyltransferase UGT76C1 finely modulates cytokinin responses via cytokinin N-glucosylation in Arabidopsis thaliana’, *Plant Physiology and Biochemistry*, vol. 65, pp. 9–16, Apr. 2013, doi: 10.1016/J.PLAPHY.2013.01.012.

[95] J. Wang, X.-M. Ma, M. Kojima, H. Sakakibara, and B.-K. Hou, ‘N-Glucosyltransferase UGT76C2 is Involved in Cytokinin Homeostasis and Cytokinin Response in Arabidopsis thaliana’, *Plant and Cell Physiology*, vol. 52, no. 12, pp. 2200–2213, Dec. 2011, doi: 10.1093/pcp/pcr152.

[96] B. Brzobohatý *et al.*, ‘Release of active cytokinin by a beta-glucosidase localized to the maize root meristem’, *Science*, vol. 262, no. 5136, pp. 1051–1054, Nov. 1993, doi: 10.1126/science.8235622.

[97] S.-H. Jin, X.-M. Ma, M. Kojima, H. Sakakibara, Y.-W. Wang, and B.-K. Hou, ‘Overexpression of glucosyltransferase UGT85A1 influences trans-zeatin homeostasis and trans-zeatin responses likely through O-glucosylation’, *Planta*, vol. 237, no. 4, pp. 991–999, Apr. 2013, doi: 10.1007/s00425-012-1818-4.

[98] E. Zažímalová, A. S. Murphy, H. Yang, K. Hoyerová, and P. Hošek, ‘Auxin Transporters—Why So Many?’, *Cold Spring Harb Perspect Biol*, vol. 2, no. 3, p. a001552, Jan. 2010, doi: 10.1101/cshperspect.a001552.

[99] G. J. Mitchison, D. E. Hanke, and A. R. Sheldrake, ‘The Polar Transport of Auxin and Vein Patterns in Plants [and Discussion]’, *Philosophical Transactions of the Royal Society B: Biological Sciences*, vol. 295, no. 1078, pp. 461–471, Oct. 1981, doi: 10.1098/rstb.1981.0154.

[100] A.-G. Rolland-Lagan, P. Federl, and P. Prusinkiewicz, ‘Reviewing models of auxin canalisation in the context of vein pattern formation in Arabidopsis leaves’, 2004.

[101] F. G. Feugier, A. Mochizuki, and Y. Iwasa, ‘Self-organization of the vascular system in plant leaves: Inter-dependent dynamics of auxin flux and carrier proteins’, *Journal of Theoretical Biology*, vol. 236, no. 4, pp. 366–375, Oct. 2005, doi: 10.1016/j.jtbi.2005.03.017.

[102] R. M. H. Merks, Y. V. de Peer, D. Inzé, and G. T. S. Beemster, ‘Canalization without flux sensors: a traveling-wave hypothesis’, *Trends in Plant Science*, vol. 12, no. 9, pp. 384–390, Sep. 2007, doi: 10.1016/j.tplants.2007.08.004.

[103] R. S. Smith, S. Guyomarc’h, T. Mandel, D. Reinhardt, C. Kuhlemeier, and P. Prusinkiewicz, ‘A plausible model of phyllotaxis’, *PNAS*, vol. 103, no. 5, pp. 1301–1306, Jan. 2006, doi: 10.1073/pnas.0510457103.

[104] H. Jonsson, M. G. Heisler, B. E. Shapiro, E. M. Meyerowitz, and E. Mjolsness, ‘An auxin-driven polarized transport model for phyllotaxis’, *Proceedings of the National Academy of Sciences*, vol. 103, no. 5, pp. 1633–1638, Jan. 2006, doi: 10.1073/pnas.0509839103.

[105] P. Krupinski and H. Jönsson, ‘Modeling Auxin-regulated Development’, *Cold Spring Harb Perspect Biol*, vol. 2, no. 2, p. a001560, Jan. 2010, doi: 10.1101/cshperspect.a001560.

[106] H. Lodish, A. Berk, S. L. Zipursky, P. Matsudaira, D. Baltimore, and J. Darnell, ‘Transport across Cell Membranes’, in *Molecular Cell Biology*, 4th edition., H. Lodish, Ed. New York: W.H. Freeman, 2000.

[107] A. Fick, ‘Ueber Diffusion’, *Annalen der Physik*, vol. 170, no. 1, pp. 59–86, 1855, doi: 10.1002/andp.18551700105.

[108] A. Paul, T. Laurila, V. Vuorinen, and S. V. Divinski, ‘Fick’s Laws of Diffusion’, in *Thermodynamics, Diffusion and the Kirkendall Effect in Solids*, A. Paul, T. Laurila, V. Vuorinen, and S. V. Divinski, Eds. Cham: Springer International Publishing, 2014, pp. 115–139.

[109] A. Cedzich, H. Stransky, B. Schulz, and W. B. Frommer, ‘Characterization of Cytokinin and Adenine Transport in Arabidopsis Cell Cultures’, 2008, doi: 10.1104/pp.108.128454.

[110] S. el-Showk *et al.*, ‘Parsimonious Model of Vascular Patterning Links Transverse Hormone Fluxes to Lateral Root Initiation: Auxin Leads the Way, while Cytokinin Levels Out’, *PLOS Computational Biology*, vol. 11, no. 10, p. e1004450, Oct. 2015, doi: 10.1371/journal.pcbi.1004450.

[111] S. Moore *et al.*, ‘Spatiotemporal modelling of hormonal crosstalk explains the level and patterning of hormones and gene expression in Arabidopsis thaliana wild-type and mutant roots’, *New Phytologist*, vol. 207, no. 4, pp. 1110–1122, 2015, doi: 10.1111/nph.13421.
[112] A. Osugi and H. Sakakibara, ‘Q&A: How do plants respond to cytokinins and what is their importance?’, *BMC Biol*, vol. 13, Nov. 2015, doi: 10.1186/s12915-015-0214-5.
[113] P. H. Rubery and A. R. Sheldrake, ‘Effect of pH and Surface Charge on Cell Uptake of Auxin’, *Nature New Biology*, vol. 244, no. 139, pp. 285–288, Aug. 1973, doi: 10.1038/newbio244285a0.
[114] J. A. Raven, ‘Transport of Indoleacetic Acid in Plant Cells in Relation to pH and Electrical Potential Gradients, and its Significance for Polar IAA Transport’, *New Phytologist*, vol. 74, no. 2, pp. 163–172, 1975, doi: 10.1111/j.1469-8137.1975.tb02602.x.
[115] M. Estelle, ‘Polar Auxin Transport: New Support for an Old Model’, *The Plant Cell*, vol. 10, no. 11, pp. 1775–1778, Nov. 1998, doi: 10.1105/tpc.10.11.1775.
[116] A. Steinacher, O. Leyser, and R. H. Clayton, ‘A computational model of auxin and pH dynamics in a single plant cell’, *Journal of Theoretical Biology*, vol. 296, pp. 84–94, Mar. 2012, doi: 10.1016/j.jtbi.2011.11.020.
[117] T. Nordey, M. Léchaudel, M. Génard, and J. Joas, ‘Factors affecting ethylene and carbon dioxide concentrations during ripening: Incidence on final dry matter, total soluble solids content and acidity of mango fruit’, *Journal of Plant Physiology*, vol. 196–197, pp. 70–78, Jun. 2016, doi: 10.1016/j.jplph.2016.03.008.
[118] L. Michaelis and M. L. Menten, ‘Die Kinetik der Invertinwirkung’, *Biochemische Zeitschrift*, vol. 49, pp. 333–369, 1913.
[119] K. A. Johnson and R. S. Goody, ‘The Original Michaelis Constant: Translation of the 1913 Michaelis-Menten Paper’, *Biochemistry*, vol. 50, no. 39, pp. 8264–8269, Oct. 2011, doi: 10.1021/bi201284u.
[120] P. L. Pedersen, ‘Transport ATPases into the year 2008: a brief overview related to types, structures, functions and roles in health and disease’, *J Bioenerg Biomembr*, vol. 39, no. 5, pp. 349–355, Dec. 2007, doi: 10.1007/s10863-007-9123-9.
[121] S. Wilkens, ‘Structure and mechanism of ABC transporters’, *F1000Prime Rep*, vol. 7, Feb. 2015, doi: 10.12703/P7-14.
[122] J. Kang *et al.*, ‘Plant ABC Transporters’, *Arabidopsis Book*, vol. 9, Dec. 2011, doi: 10.1199/tab.0153.
[123] N. Shitan *et al.*, ‘Involvement of CjMDR1, a plant multidrug-resistance-type ATP-binding cassette protein, in alkaloid transport in Coptis japonica’, *Proc. Natl. Acad. Sci. U.S.A.*, vol. 100, no. 2, pp. 751–756, Jan. 2003, doi: 10.1073/pnas.0134257100.
[124] M. Dean, A. Rzhetsky, and R. Allikmets, ‘The Human ATP-Binding Cassette (ABC) Transporter Superfamily’, *Genome Res.*, vol. 11, no. 7, pp. 1156–1166, Jan. 2001, doi: 10.1101/gr.184901.
[125] P. J. Verrier *et al.*, ‘Plant ABC proteins – a unified nomenclature and updated inventory’, *Trends in Plant Science*, vol. 13, no. 4, pp. 151–159, Apr. 2008, doi: 10.1016/j.tplants.2008.02.001.
[126] B. Noh, A. S. Murphy, and E. P. Spalding, ‘Multidrug resistance-like genes of Arabidopsis required for auxin transport and auxin-mediated development’, *Plant Cell*, vol. 13, no. 11, pp. 2441–2454, Nov. 2001, doi: 10.1105/tpc.010350.
[127] M. Geisler *et al.*, ‘Cellular efflux of auxin catalyzed by the Arabidopsis MDR/PGP transporter AtPGP1’, *The Plant Journal*, vol. 44, no. 2, pp. 179–194, 2005, doi: 10.1111/j.1365-313X.2005.02519.x.
[128] H. Yang and A. S. Murphy, ‘Functional expression and characterization of Arabidopsis ABCB, AUX 1 and PIN auxin transporters in Schizosaccharomyces pombe’, *The Plant Journal*, vol. 59, no. 1, pp. 179–191, 2009, doi: 10.1111/j.1365-313X.2009.03856.x.
[129] D. Santelia *et al.*, ‘MDR-like ABC transporter AtPGP4 is involved in auxin-mediated lateral root and root hair development’, *FEBS Lett.*, vol. 579, no. 24, pp. 5399–5406, Oct. 2005, doi: 10.1016/j.febslet.2005.08.061.
[130] B. Titapiwatanakun *et al.*, ‘ABCB19/PGP19 stabilises PIN1 in membrane microdomains in Arabidopsis’, *The Plant Journal*, vol. 57, no. 1, pp. 27–44, 2009, doi: 10.1111/j.1365-313X.2008.03668.x.
[131] A. Bailly, H. Yang, E. Martinoia, M. Geisler, and A. S. Murphy, ‘Plant Lessons: Exploring ABCB Functionality Through Structural Modeling’, *Front. Plant Sci.*, vol. 2, 2012, doi: 10.3389/fpls.2011.00108.
[132] K. Růžička *et al.*, ‘Arabidopsis PIS1 encodes the ABCG37 transporter of auxinic compounds including the auxin precursor indole-3-butyric acid’, *PNAS*, vol. 107, no. 23, pp. 10749–10753, Jun. 2010, doi: 10.1073/pnas.1005878107.

[133] L. C. Strader and B. Bartel, ‘The Arabidopsis PLEIOTROPIC DRUG RESISTANCE8/ABCG36 ATP Binding Cassette Transporter Modulates Sensitivity to the Auxin Precursor Indole-3-Butyric Acid’, *The Plant Cell*, vol. 21, no. 7, pp. 1992–2007, Jul. 2009, doi: 10.1105/tpc.109.065821.
[134] T. Kuromori *et al.*, ‘ABC transporter AtABCG25 is involved in abscisic acid transport and responses’, *PNAS*, vol. 107, no. 5, pp. 2361–2366, Feb. 2010, doi: 10.1073/pnas.0912516107.
[135] J. Kang *et al.*, ‘PDR-type ABC transporter mediates cellular uptake of the phytohormone abscisic acid’, *PNAS*, vol. 107, no. 5, pp. 2355–2360, Feb. 2010, doi: 10.1073/pnas.0909222107.
[136] T. Kuromori, E. Sugimoto, and K. Shinozaki, ‘Arabidopsis mutants of AtABCG22, an ABC transporter gene, increase water transpiration and drought susceptibility’, *The Plant Journal*, vol. 67, no. 5, pp. 885–894, 2011, doi: 10.1111/j.1365-313X.2011.04641.x.
[137] Y. Durán-Medina, D. Díaz-Ramírez, and N. Marsch-Martínez, ‘Cytokinins on the Move’, *Frontiers in Plant Science*, vol. 8, Feb. 2017, doi: 10.3389/fpls.2017.00146.
[138] S. A. Baldwin, J. R. Mackey, C. E. Cass, and J. D. Young, ‘Nucleoside transporters: molecular biology and implications for therapeutic development’, *Molecular Medicine Today*, vol. 5, no. 5, pp. 216–224, May 1999, doi: 10.1016/S1357-4310(99)01459-8.
[139] C. Girke, M. Daumann, S. Niopek-Witz, and T. Möhlmann, ‘Nucleobase and nucleoside transport and integration into plant metabolism’, *Front. Plant Sci.*, vol. 5, 2014, doi: 10.3389/fpls.2014.00443.
[140] J. A. Thorn and S. M. Jarvis, ‘Adenosine transporters’, *General Pharmacology: The Vascular System*, vol. 27, no. 4, pp. 613–620, Jun. 1996, doi: 10.1016/0306-3623(95)02053-5.
[141] J. Li and D. Wang, ‘Cloning and in vitro expression of the cDNA encoding a putative nucleoside transporter from Arabidopsis thaliana’, *Plant Science*, vol. 157, no. 1, pp. 23–32, Aug. 2000, doi: 10.1016/S0168-9452(00)00261-2.
[142] A. E. King, M. A. Ackley, C. E. Cass, J. D. Young, and S. A. Baldwin, ‘Nucleoside transporters: from scavengers to novel therapeutic targets’, *Trends in Pharmacological Sciences*, vol. 27, no. 8, pp. 416–425, Aug. 2006, doi: 10.1016/j.tips.2006.06.004.
[143] J. D. Young, S. Y. M. Yao, J. M. Baldwin, C. E. Cass, and S. A. Baldwin, ‘The human concentrative and equilibrative nucleoside transporter families, SLC28 and SLC29’, *Molecular Aspects of Medicine*, vol. 34, no. 2, pp. 529–547, Apr. 2013, doi: 10.1016/j.mam.2012.05.007.
[144] J. Sun *et al.*, ‘Arabidopsis SOI33/AtENT8 Gene Encodes a Putative Equilibrative Nucleoside Transporter That Is Involved in Cytokinin Transport In Planta’, *Journal of Integrative Plant Biology*, vol. 47, no. 5, pp. 588–603, May 2005, doi: 10.1111/j.1744-7909.2005.00104.x.
[145] T. Möhlmann, Z. Mezher, G. Schwerdtfeger, and H. E. Neuhaus, ‘Characterisation of a concentrative type of adenosine transporter from Arabidopsis thaliana (ENT1,At)’, *FEBS Letters*, vol. 509, no. 3, pp. 370–374, Dec. 2001, doi: 10.1016/S0014-5793(01)03195-7.
[146] G. Li, K. Liu, S. A. Baldwin, and D. Wang, ‘Equilibrative Nucleoside Transporters of Arabidopsis thaliana: cDNA cloning, expression pattern, and analysis of transport activities.’, *J. Biol. Chem.*, vol. 278, no. 37, pp. 35732–35742, Dec. 2003, doi: 10.1074/jbc.M304768200.
[147] A. Wormit, M. Traub, M. Flörchinger, H. E. Neuhaus, and T. Möhlmann, ‘Characterization of three novel members of the Arabidopsis thaliana equilibrative nucleoside transporter (ENT) family’, *Biochem J*, vol. 383, no. 1, pp. 19–26, Oct. 2004, doi: 10.1042/BJ20040389.
[148] N. Hirose, K. Takei, T. Kuroha, T. Kamada-Nobusada, H. Hayashi, and H. Sakakibara, ‘Regulation of cytokinin biosynthesis, compartmentalization and translocation’, *J Exp Bot*, vol. 59, no. 1, pp. 75–83, Jan. 2008, doi: 10.1093/jxb/erm157.
[149] N. Hirose, N. Makita, T. Yamaya, and H. Sakakibara, ‘Functional Characterization and Expression Analysis of a Gene, OsENT2, Encoding an Equilibrative Nucleoside Transporter in Rice Suggest a Function in Cytokinin Transport’, *Plant Physiol*, vol. 138, no. 1, pp. 196–206, May 2005, doi: 10.1104/pp.105.060137.
[150] B. Gillissen *et al.*, ‘A New Family of High-Affinity Transporters for Adenine, Cytosine, and Purine Derivatives in Arabidopsis’, *Plant Cell*, vol. 12, no. 2, pp. 291–300, Feb. 2000.
[151] J. G. Jelesko, ‘An expanding role for purine uptake permease-like transporters in plant secondary metabolism’, *Front. Plant Sci.*, vol. 3, 2012, doi: 10.3389/fpls.2012.00078.
[152] S. B. Hildreth *et al.*, ‘Tobacco nicotine uptake permease (NUP1) affects alkaloid metabolism’, *Proc Natl Acad Sci U S A*, vol. 108, no. 44, pp. 18179–18184, Nov. 2011, doi: 10.1073/pnas.1108620108.
[153] L. Bürkle *et al.*, ‘Transport of cytokinins mediated by purine transporters of the PUP family expressed in phloem, hydathodes, and pollen of Arabidopsis’, *The Plant Journal*, vol. 34, no. 1, pp. 13–26, Apr. 2003, doi: 10.1046/j.1365-313X.2003.01700.x.

[154] Z. Qi and L. Xiong, ‘Characterization of a Purine Permease Family Gene OsPUP7 Involved in Growth and Development Control in Rice’, *Journal of Integrative Plant Biology*, vol. 55, no. 11, pp. 1119–1135, Nov. 2013, doi: 10.1111/jipb.12101.
[155] E. Zürcher, J. Liu, M. di Donato, M. Geisler, and B. Müller, ‘Plant development regulated by cytokinin sinks’, *Science*, vol. 353, no. 6303, pp. 1027–1030, 2016.
[156] E. Zürcher, D. Tavor-Deslex, D. Lituiev, K. Enkerli, P. T. Tarr, and B. Müller, ‘A Robust and Sensitive Synthetic Sensor to Monitor the Transcriptional Output of the Cytokinin Signaling Network in Planta’, *Plant Physiol*, vol. 161, no. 3, pp. 1066–1075, Mar. 2013, doi: 10.1104/pp.112.211763.
[157] D. Ko *et al.*, ‘Arabidopsis ABCG14 is essential for the root-to-shoot translocation of cytokinin.’, *Proceedings of the National Academy of Sciences of the United States of America*, vol. 111, no. 19, pp. 7150–5, May 2014, doi: 10.1073/pnas.1321519111.
[158] R. Le Hir *et al.*, ‘ABCG9, ABCG11 and ABCG14 ABC transporters are required for vascular development in Arabidopsis’, *The Plant Journal*, vol. 76, no. 5, pp. 811–824, 2013, doi: 10.1111/tpj.12334.
[159] K. Zhang *et al.*, ‘Arabidopsis ABCG14 protein controls the acropetal translocation of root-synthesized cytokinins’, *Nature Communications*, vol. 5, pp. 1–12, Feb. 2014, doi: 10.1038/ncomms4274.
[160] K. Hoyerová *et al.*, ‘Efficiency of different methods of extraction and purification of cytokinins’, *Phytochemistry*, vol. 67, no. 11, pp. 1151–1159, Jun. 2006, doi: 10.1016/j.phytochem.2006.03.010.
[161] D. J. Armstrong, W. J. Burrows, P. K. Evans, and F. Skoog, ‘Isolation of cytokinins from tRNA’, *Biochemical and Biophysical Research Communications*, vol. 37, no. 3, pp. 451–456, Oct. 1969, doi: 10.1016/0006-291X(69)90936-X.
[162] N. R. Crouch and J. Van Staden, ‘Extraction of Cytokinin Nucleotides with Bieleski Solvents: Is the Inconvenience Warranted?’, *Journal of Plant Physiology*, vol. 140, no. 3, pp. 378–380, Aug. 1992, doi: 10.1016/S0176-1617(11)81097-7.
[163] M. Kojima *et al.*, ‘Highly Sensitive and High-Throughput Analysis of Plant Hormones Using MS-Probe Modification and Liquid Chromatography–Tandem Mass Spectrometry: An Application for Hormone Profiling in Oryza sativa’, *Plant Cell Physiol*, vol. 50, no. 7, pp. 1201–1214, Jul. 2009, doi: 10.1093/pcp/pcp057.
[164] M. Laloue, C. Terrine, and M. Gawer, ‘Cytokinins: Formation of the nucleoside-5′-triphosphate in tobacco and Acer cells’, *FEBS Letters*, vol. 46, no. 1, pp. 45–50, Sep. 1974, doi: 10.1016/0014-5793(74)80331-5.
[165] R. L. Bieleski, ‘The problem of halting enzyme action when extracting plant tissues’, *Analytical Biochemistry*, vol. 9, no. 4, pp. 431–442, Dec. 1964, doi: 10.1016/0003-2697(64)90204-0.
[166] P. Redig, T. Schmulling, and H. V. Onckelen, ‘Analysis of Cytokinin Metabolism in ipt Transgenic Tobacco by Liquid Chromatography-Tandem Mass Spectrometry’, *Plant Physiology*, vol. 112, no. 1, pp. 141–148, Sep. 1996, doi: 10.1104/pp.112.1.141.
[167] S. Acharya, A. Földesi, and J. Chattopadhyaya, ‘The pKa of the Internucleotidic 2‘-Hydroxyl Group in Diribonucleoside (3‘→5‘) Monophosphates’, *J. Org. Chem.*, vol. 68, no. 5, pp. 1906–1910, Mar. 2003, doi: 10.1021/jo026545o.
[168] P. C. Bevilacqua, T. S. Brown, S. Nakano, and R. Yajima, ‘Catalytic roles for proton transfer and protonation in ribozymes’, *Biopolymers*, vol. 73, no. 1, pp. 90–109, 2004, doi: 10.1002/bip.10519.
[169] P. I. Dobrev and M. Kamínek, ‘Fast and efficient separation of cytokinins from auxin and abscisic acid and their purification using mixed-mode solid-phase extraction’, *Journal of Chromatography A*, vol. 950, no. 1–2, pp. 21–29, Mar. 2002, doi: 10.1016/S0021-9673(02)00024-9.
[170] P. Thaplyal and P. C. Bevilacqua, ‘Experimental Approaches for Measuring pKa’s in RNA and DNA’, *Methods Enzymol*, vol. 549, pp. 189–219, 2014, doi: 10.1016/B978-0-12-801122-5.00009-X.
[171] G. Guinn and D. L. Brummett, ‘Solid-phase extraction of cytokinins from aqueous solutions with C18 cartridges and their use in a rapid purification procedure’, *Plant Growth Regul*, vol. 9, no. 4, pp. 305–314, Nov. 1990, doi: 10.1007/BF00024916.
[172] R. O. Morris, J. B. Zaerr, and R. W. Chapman, ‘Trace enrichment of cytokinins from Douglas-fir xylem extrudate’, *Planta*, vol. 131, no. 3, pp. 271–274, Jan. 1976, doi: 10.1007/BF00385425.
[173] P. I. Dobrev, L. Havlíček, M. Vágner, J. Malbeck, and M. Kamínek, ‘Purification and determination of plant hormones auxin and abscisic acid using solid phase extraction and two-dimensional high performance liquid chromatography’, *Journal of Chromatography A*, vol. 1075, no. 1, pp. 159–166, May 2005, doi: 10.1016/j.chroma.2005.02.091.

[174] R. Vaňková *et al.*, ‘Comparison of oriented and random antibody immobilization in immunoaffinity chromatography of cytokinins’, *Journal of Chromatography A*, vol. 811, no. 1, pp. 77–84, Jun. 1998, doi: 10.1016/S0021-9673(98)00210-6.
[175] P. Ulvskov, T. H. Nielsen, P. Seiden, and J. Marcussen, ‘Cytokinins and leaf development in sweet pepper (Capsicum annuum L.)’, *Planta*, vol. 188, no. 1, pp. 70–77, Mar. 1992, doi: 10.1007/BF01160714.
[176] E. W. Weiler, ‘Radioimmunoassays for trans-zeatin and related cytokinins’, *Planta*, vol. 149, no. 2, pp. 155–162, Jul. 1980, doi: 10.1007/BF00380877.
[177] T. L. Wang, S. K. Cook, and J. P. Knox, ‘Monoclonal antibodies for the analysis and purification of isopentenyladenine cytokinins’, *Phytochemistry*, vol. 26, no. 9, pp. 2447–2452, Jan. 1987, doi: 10.1016/S0031-9422(00)83851-0.
[178] M. Faiss, J. Zalubilová, M. Strnad, and T. Schmülling, ‘Conditional transgenic expression of the ipt gene indicates a function for cytokinins in paracrine signaling in whole tobacco plants’, *The Plant Journal*, vol. 12, no. 2, pp. 401–415, 1997, doi: 10.1046/j.1365-313X.1997.12020401.x.
[179] T. Werner, J. Hanuš, J. Holub, T. Schmülling, H. V. Onckelen, and M. Strnad, ‘New cytokinin metabolites in IPT transgenic Arabidopsis thaliana plants’, *Physiologia Plantarum*, vol. 118, no. 1, pp. 127–137, 2003, doi: 10.1034/j.1399-3054.2003.00094.x.
[180] O. Novák, P. Tarkowski, D. Tarkowská, K. Doležal, R. Lenobel, and M. Strnad, ‘Quantitative analysis of cytokinins in plants by liquid chromatography–single-quadrupole mass spectrometry’, *Analytica Chimica Acta*, vol. 480, no. 2, pp. 207–218, Mar. 2003, doi: 10.1016/S0003-2670(03)00025-4.
[181] W. Jordi *et al.*, ‘Increased cytokinin levels in transgenic PSAG12-IPT tobacco plants have large direct and indirect effects on leaf senescence, photosynthesis and N partitioning’, *Plant, Cell and Environment*, vol. 23, no. 3, pp. 279–289, Mar. 2000, doi: 10.1046/j.1365-3040.2000.00544.x.
[182] J. A. van Rhijn, H. H. Heskamp, E. Davelaar, W. Jordi, M. S. Leloux, and U. A. T. Brinkman, ‘Quantitative determination of glycosylated and aglycon isoprenoid cytokinins at sub-picomolar levels by microcolumn liquid chromatography combined with electrospray tandem mass spectrometry’, *Journal of Chromatography A*, vol. 929, no. 1, pp. 31–42, Sep. 2001, doi: 10.1016/S0021-9673(01)01134-7.
[183] C. Åstot, K. Dolezal, T. Moritz, and G. Sandberg, ‘Precolumn derivatization and capillary liquid chromatographic/frit-fast atom bombardment mass spectrometric analysis of cytokinins in Arabidopsis thaliana’, *Journal of Mass Spectrometry*, vol. 33, no. 9, pp. 892–902, 1998, doi: 10.1002/(SICI)1096-9888(199809)33:9<892::AID-JMS701>3.0.CO;2-N.
[184] A. C. Moser and D. S. Hage, ‘Immunoaffinity chromatography: an introduction to applications and recent developments’, *Bioanalysis*, vol. 2, no. 4, pp. 769–790, Apr. 2010, doi: 10.4155/bio.10.31.
[185] A. A. Kortt, G. W. Oddie, P. Iliades, L. C. Gruen, and P. J. Hudson, ‘Nonspecific Amine Immobilization of Ligand Can Be a Potential Source of Error in BIAcore Binding Experiments and May Reduce Binding Affinities’, *Analytical Biochemistry*, vol. 253, no. 1, pp. 103–111, Nov. 1997, doi: 10.1006/abio.1997.2333.
[186] X. Pan and X. Wang, ‘Profiling of plant hormones by mass spectrometry’, *Journal of Chromatography B*, vol. 877, no. 26, pp. 2806–2813, Sep. 2009, doi: 10.1016/j.jchromb.2009.04.024.
[187] C. Birkemeyer, A. Kolasa, and J. Kopka, ‘Comprehensive chemical derivatization for gas chromatography–mass spectrometry-based multi-targeted profiling of the major phytohormones’, *Journal of Chromatography A*, vol. 993, no. 1, pp. 89–102, Apr. 2003, doi: 10.1016/S0021-9673(03)00356-X.
[188] L. M. S. Palni, R. E. Summons, and D. S. Letham, ‘Mass Spectrometric Analysis of Cytokinins in Plant Tissues: V. Identification of the Cytokinin Complex of Datura Innoxia Crown Gall Tissue’, *Plant Physiology*, vol. 72, no. 3, pp. 858–863, Jul. 1983, doi: 10.1104/pp.72.3.858.
[189] H. Soejima, T. Sugiyama, and K. Ishihara, ‘Changes in Cytokinin Activities and Mass Spectrometric Analysis of Cytokinins in Root Exudates of Rice Plant (Oryza sativa L.) : Comparison between Cultivars Nipponbare and Akenohoshi’, *Plant Physiology*, vol. 100, no. 4, pp. 1724–1729, Dec. 1992, doi: 10.1104/pp.100.4.1724.
[190] B. Nicander, P. O. Bjorkman, and E. Tillberg, ‘Identification of an N-Glucoside of cis-Zeatin from Potato Tuber Sprouts’, *Plant Physiology*, vol. 109, no. 2, pp. 513–516, Oct. 1995, doi: 10.1104/pp.109.2.513.
[191] J. Engelberth, E. A. Schmelz, H. T. Alborn, Y. J. Cardoza, J. Huang, and J. H. Tumlinson, ‘Simultaneous quantification of jasmonic acid and salicylic acid in plants by vapor-phase extraction and gas chromatography-chemical ionization-mass spectrometry’, *Analytical Biochemistry*, vol. 312, no. 2, pp. 242–250, Jan. 2003, doi: 10.1016/S0003-2697(02)00466-9.

[192] E. A. Schmelz *et al.*, ‘Simultaneous analysis of phytohormones, phytotoxins, and volatile organic compounds in plants’, *Proceedings of the National Academy of Sciences*, vol. 100, no. 18, pp. 10552–10557, Sep. 2003, doi: 10.1073/pnas.1633615100.

[193] E. A. Schmelz, J. Engelberth, J. H. Tumlinson, A. Block, and H. T. Alborn, ‘The use of vapor phase extraction in metabolic profiling of phytohormones and other metabolites’, *The Plant Journal*, vol. 39, no. 5, pp. 790–808, Sep. 2004, doi: 10.1111/j.1365-313X.2004.02168.x.

[194] N. Hashimoto, T. Aoyama, and T. Shioiri, ‘New Methods and Reagents in Organic Synthesis. 14. A Simple Efficient Preparation of Methyl Esters with Trimethylsilyldiazomethane ($TMSCHN_2$) and Its Application to Gas Chromatographic Analysis of Fatty Acids’, *Chemical & Pharmaceutical Bulletin*, vol. 29, no. 5, pp. 1475–1478, 1981, doi: 10.1248/cpb.29.1475.

[195] Y.-K. Chong, C.-C. Ho, S.-Y. Leung, S. K. P. Lau, and P. C. Y. Woo, ‘Clinical Mass Spectrometry in the Bioinformatics Era: A Hitchhiker’s Guide’, *Comput Struct Biotechnol J*, vol. 16, pp. 316–334, Aug. 2018, doi: 10.1016/j.csbj.2018.08.003.

[196] F. Du, G. Ruan, and H. Liu, ‘Analytical methods for tracing plant hormones’, *Anal Bioanal Chem*, vol. 403, no. 1, pp. 55–74, Apr. 2012, doi: 10.1007/s00216-011-5623-x.

[197] E. C. Horning *et al.*, ‘Development and use of analytical systems based on mass spectrometry.’, *Clinical Chemistry*, vol. 23, no. 1, pp. 13–21, Jan. 1977.

[198] K. Gamoh, H. Abe, K. Shimada, and S. Takatsuto, ‘Liquid Chromatography/Mass Spectrometry with Atmospheric Pressure Chemical Ionization of Free Brassinosteroids’, *Rapid Communications in Mass Spectrometry*, vol. 10, no. 8, pp. 903–906, 1996, doi: 10.1002/(SICI)1097-0231(19960610)10:8<903::AID-RCM564>3.0.CO;2-2.

[199] D. R. Stoll and P. W. Carr, ‘Two-Dimensional Liquid Chromatography: A State of the Art Tutorial’, *Anal. Chem.*, vol. 89, no. 1, pp. 519–531, Jan. 2017, doi: 10.1021/acs.analchem.6b03506.

[200] X. Pan, R. Welti, and X. Wang, ‘Simultaneous quantification of major phytohormones and related compounds in crude plant extracts by liquid chromatography–electrospray tandem mass spectrometry’, *Phytochemistry*, vol. 69, no. 8, pp. 1773–1781, May 2008, doi: 10.1016/j.phytochem.2008.02.008.

[201] S. D. S. Chiwocha *et al.*, ‘A method for profiling classes of plant hormones and their metabolites using liquid chromatography-electrospray ionization tandem mass spectrometry: an analysis of hormone regulation of thermodormancy of lettuce (Lactuca sativa L.) seeds’, *The Plant Journal*, vol. 35, no. 3, pp. 405–417, 2003, doi: 10.1046/j.1365-313X.2003.01800.x.

[202] E. Prinsen, P. Redig, H. A. V. Onckelen, W. V. Dongen, and E. L. Esmans, ‘Quantitative analysis of cytokinins by electrospray tandem mass spectrometry’, *Rapid Communications in Mass Spectrometry*, vol. 9, no. 10, pp. 948–953, 1995, doi: 10.1002/rcm.1290091016.

[203] E. Prinsen, W. Van Dongen, E. L. Esmans, and H. A. Van Onckelen, ‘Micro and capillary liquid chromatography–tandem mass spectrometry: a new dimension in phytohormone research’, *Journal of Chromatography A*, vol. 826, no. 1, pp. 25–37, Nov. 1998, doi: 10.1016/S0021-9673(98)00763-8.

[204] L. Corbesier *et al.*, ‘Cytokinin levels in leaves, leaf exudate and shoot apical meristem of Arabidopsis thaliana during floral transition’, *J Exp Bot*, vol. 54, no. 392, pp. 2511–2517, Nov. 2003, doi: 10.1093/jxb/erg276.

[205] V. S. Morozova, A. I. Levashova, and S. A. Eremin, ‘Determination of pesticides by enzyme immunoassay’, *J Anal Chem*, vol. 60, no. 3, pp. 202–217, Mar. 2005, doi: 10.1007/s10809-005-0075-0.

[206] J. Zhao *et al.*, ‘Comparison between conventional indirect competitive enzyme-linked immunosorbent assay (icELISA) and simplified icELISA for small molecules’, *Analytica Chimica Acta*, vol. 571, no. 1, pp. 79–85, Jun. 2006, doi: 10.1016/j.aca.2006.04.060.

[207] L. Sáenz, L. H. Jones, C. Oropeza, D. Vláčil, and M. Strnad, ‘Endogenous isoprenoid and aromatic cytokinins in different plant parts of Cocos nucifera (L.)’, *Plant Growth Regulation*, vol. 39, no. 3, pp. 205–215, Mar. 2003, doi: 10.1023/A:1022851012878.

[208] T. M. Annesley, ‘It’s about the Journey, Not the Destination: The Birth of Radioimmunoassay’, *Clinical Chemistry*, vol. 56, no. 4, pp. 671–672, Apr. 2010, doi: 10.1373/clinchem.2010.142950.

[209] R. S. Yalow and S. A. Berson, ‘Immunoassay of Endogenous Plasma Insulin in Man’, *J Clin Invest*, vol. 39, no. 7, pp. 1157–1175, Jul. 1960.

[210] R. D. Grange, J. P. Thompson, and D. G. Lambert, ‘Radioimmunoassay, enzyme and non-enzyme-based immunoassays’, *British Journal of Anaesthesia*, vol. 112, no. 2, pp. 213–216, Feb. 2014, doi: 10.1093/bja/aet293.

[211] D. Ernst, W. Schäfer, and D. Oesterhelt, ‘Isolation and quantitation of isopentenyladenosine in an anise cell culture by single-ion monitoring, radioimmunoassay and bioassay’, *Planta*, vol. 159, no. 3, pp. 216–221, Nov. 1983, doi: 10.1007/BF00397527.
[212] J. Badenoch-Jones, D. S. Letham, C. W. Parker, and B. G. Rolfe, ‘Quantitation of Cytokinins in Biological Samples Using Antibodies Against Zeatin Riboside’, *Plant Physiol*, vol. 75, no. 4, pp. 1117–1125, Aug. 1984.
[213] Y. Liang, M.-P. Zhao, and H.-W. Liu, ‘Research Progress in Cytokinins Analysis’, *Chinese Journal of Analytical Chemistry*, vol. 37, no. 8, pp. 1232–1239, Aug. 2009, doi: 10.1016/S1872-2040(08)60124-3.
[214] D. Nedvěd, ‘Transport cytokininů přes plasmatickou membránu’, bachelor, Charles University, Prague, 2018.
[215] P. Klíma, ‘Mechanism of cytokinin ransport across plasma membrane and their metabolism in tobacco BY-2 cultured cells’, Ph.D., Charles University, Prague, 2011.
[216] T. Nagata, Y. Nemoto, and S. Hasezawa, ‘Tobacco BY-2 Cell Line as the “HeLa” Cell in the Cell Biology of Higher Plants’, *International Review of Cytology*, vol. 132, pp. 1–30, Jan. 1992, doi: 10.1016/S0074-7696(08)62452-3.
[217] T. Murashige and F. Skoog, ‘A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures’, *Physiologia Plantarum*, vol. 15, no. 3, pp. 473–497, 1962, doi: 10.1111/j.1399-3054.1962.tb08052.x.
[218] O. L. Gamborg, R. A. Miller, and K. Ojima, ‘Nutrient requirements of suspension cultures of soybean root cells’, *Experimental Cell Research*, vol. 50, no. 1, pp. 151–158, Apr. 1968, doi: 10.1016/0014-4827(68)90403-5.
[219] M. Pernisová *et al.*, ‘Cytokinins modulate auxin-induced organogenesis in plants via regulation of the auxin efflux’, *PNAS*, vol. 106, no. 9, pp. 3609–3614, Mar. 2009, doi: 10.1073/pnas.0811539106.
[220] M. Kubo and T. Kakimoto, ‘The CYTOKININ-HYPERSENSITIVE genes of Arabidopsis negatively regulate the cytokinin-signaling pathway for cell division and chloroplast development’, *The Plant Journal*, vol. 23, no. 3, pp. 385–394, 2000, doi: 10.1046/j.1365-313x.2000.00796.x.
[221] A. Delbarre, P. Muller, V. Imhoff, and J. Guern, ‘Comparison of mechanisms controlling uptake and accumulation of 2,4-dichlorophenoxy acetic acid, naphthalene-1-acetic acid, and indole-3-acetic acid in suspension-cultured tobacco cells’, *Planta*, vol. 198, no. 4, pp. 532–541, Apr. 1996, doi: 10.1007/BF00262639.
[222] J. Petrášek, M. Elčkner, D. A. Morris, and E. Zažímalová, ‘Auxin efflux carrier activity and auxin accumulation regulate cell division and polarity in tobacco cells’, *Planta*, vol. 216, no. 2, pp. 302–308, Dec. 2002, doi: 10.1007/s00425-002-0845-y.
[223] J. Petrášek, A. Černá, K. Schwarzerová, M. Elčkner, D. A. Morris, and E. Zažímalová, ‘Do Phytotropins Inhibit Auxin Efflux by Impairing Vesicle Traffic?’, *Plant Physiology*, vol. 131, no. 1, pp. 254–263, Jan. 2003, doi: 10.1104/pp.012740.
[224] D. Vivian and J. E. Polli, ‘Mechanistic Interpretation of Conventional Michaelis-Menten Parameters in a Transporter System’, *Eur J Pharm Sci*, vol. 64, pp. 44–52, Nov. 2014, doi: 10.1016/j.ejps.2014.08.007.
[225] D. Seifertová *et al.*, ‘Characterization of transmembrane auxin transport in Arabidopsis suspension-cultured cells’, *Journal of Plant Physiology*, vol. 171, no. 6, pp. 429–437, Mar. 2014, doi: 10.1016/j.jplph.2013.09.026.
[226] P. Virtanen *et al.*, ‘SciPy 1.0: fundamental algorithms for scientific computing in Python’, *Nat Methods*, vol. 17, no. 3, pp. 261–272, Mar. 2020, doi: 10.1038/s41592-019-0686-2.
[227] C. Kato *et al.*, ‘Involvement of xylem sap zeatin-O-glucoside in cucumber shoot greening’, *Plant Physiology and Biochemistry*, vol. 40, no. 11, pp. 949–954, Nov. 2002, doi: 10.1016/S0981-9428(02)01458-4.
[228] E.-K. Lim and D. J. Bowles, ‘A class of plant glycosyltransferases involved in cellular homeostasis’, *EMBO J.*, vol. 23, no. 15, pp. 2915–2922, Aug. 2004, doi: 10.1038/sj.emboj.7600295.
[229] M. Šmehilová, J. Dobrůšková, O. Novák, T. Takáč, and P. Galuszka, ‘Cytokinin-Specific Glycosyltransferases Possess Different Roles in Cytokinin Homeostasis Maintenance’, *Front Plant Sci*, vol. 7, Aug. 2016, doi: 10.3389/fpls.2016.01264.
[230] E. Jiskrová *et al.*, ‘Extra- and intracellular distribution of cytokinins in the leaves of monocots and dicots’, *New Biotechnology*, vol. 33, no. 5, Part B, pp. 735–742, Sep. 2016, doi: 10.1016/j.nbt.2015.12.010.

[231] E. Bassil, A. Coku, and E. Blumwald, ‘Cellular ion homeostasis: emerging roles of intracellular NHX Na+/H+ antiporters in plant growth and development’, *J Exp Bot*, vol. 63, no. 16, pp. 5727–5740, Oct. 2012, doi: 10.1093/jxb/ers250.
[232] R. Höhner, A. Aboukila, H.-H. Kunz, and K. Venema, ‘Proton Gradients and Proton-Dependent Transport Processes in the Chloroplast’, *Front. Plant Sci.*, vol. 7, 2016, doi: 10.3389/fpls.2016.00218.
[233] J. Pittman, ‘Multiple Transport Pathways for Mediating Intracellular pH Homeostasis: The Contribution of H+/ion Exchangers’, *Front. Plant Sci.*, vol. 3, 2012, doi: 10.3389/fpls.2012.00011.
[234] G. R. Kudoyarova *et al.*, ‘Accumulation of cytokinins in roots and their export to the shoots of durum wheat plants treated with the protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP)’, *J Exp Bot*, vol. 65, no. 9, pp. 2287–2294, Jun. 2014, doi: 10.1093/jxb/eru113.
[235] I. Frébort, M. Kowalska, T. Hluska, J. Frébortová, and P. Galuszka, ‘Evolution of cytokinin biosynthesis and degradation’, *J Exp Bot*, vol. 62, no. 8, pp. 2431–2452, May 2011, doi: 10.1093/jxb/err004.
[236] P. Galuszka *et al.*, ‘Biochemical Characterization of Cytokinin Oxidases/Dehydrogenases from Arabidopsis thaliana Expressed in Nicotiana tabacum L.’, *J Plant Growth Regul*, vol. 26, no. 3, pp. 255–267, Sep. 2007, doi: 10.1007/s00344-007-9008-5.

Svoluji k zapůjčení této práce pro studijní účely a prosím, aby byla řádně vedena evidence vypůjčovatelů.

Jméno a příjmení S adresou	Číslo OP	Datum vypůjčení	Poznámka